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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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TITLE OF THE INVENTION

OLIGONUCLEOTIDE COMPLEX COMPOSITIONS AND METHODS OF USE AS GENE ALTERATION TOOLS

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ENCLOSED APPLICATION PARTS

- ☒ **Specification** — Number of Pages: 34, including two pages of claims
- ☐ **Sequence Listing** — Number of Pages:
- ☒ **Drawings** (☐ Formal; ☒ Informal) — Number of Sheets: 20
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METHOD OF PAYMENT

- ☒ A check in the amount of **$80.00** is enclosed to cover the filing fees of the Provisional application.
- ☐ The Commissioner is hereby authorized to charge **$80.00** is enclosed to cover the filing fees of the Provisional application to Deposit Account No. 50-0311, Reference No. 24028-015.
- ☒ The Commissioner is hereby authorized to charge additional fees or credit any overpayment to Deposit Account No. 50-0311, Reference No. 24028-015.

Respectfully submitted,

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Oligonucleotide Complex Compositions and Methods of Use as Gene Alteration Tools

Field of invention

The present invention relates to the use of specific complexes of oligonucleotides and their modification products as therapeutic and/or prophylactic agents for the phenotypic and/or genotypic restoration of mutated genes, genes with inborn errors and/or for switch-on and/or switch-off of targeted gene(s).

Background of Invention

Numerous genetic diseases are caused by mutations in the mammalian genome. Other sources of genetic diseases are activation of silent genes or the presence of viral genes in the mammalian genome. Several types of modifications have been found to be mutated in the genome: deletion of one or several base pairs, one or several mismatches in the sequence of the gene, insertion of one or several bases, or repeat triplet reiteration and absence of a whole or part of a gene.

Genetic diseases caused by mismatches, deletion/insertion of one or several base pairs (BP) and repeat triplet mutation in the genes include: albinism, cystic fibrosis, muscular dystrophy and atrophy, sickle cell anemia, hepatic disorders, hemophilia, Crigler-Najjar syndrome, renal tubular acidosis, β-thalassemia, atherosclerosis, Huntington's disease, spinocerebellar ataxia (type 1, 2 and 6), Machado-Joseph disease, myotonic dystrophy, Fragile X (forms A and B), and Frederich's ataxia [see Breschel et al., Human Molec. Gen. (1997) 6, 1855-1863; Kmiec, Clin. Invest. (2003) 112, 632-636].

Oligonucleotide complexes and their analogs have been employed as a potential therapeutic for the readout of genes [see McManus et al., Nat Rev Genet. (2002) 3, 737-747; Nielsen, Curr. Med. Chem. (2001) 8, 545-550; Agrawal et al., Curr. Cancer Drug Targets. (2001) 1, 197-209] and for targeted gene repair [see Kmiec, Clin. Invest. (2003) 112, 632-636].

An approach in the field of gene therapy is introduction of sequence-specific modification of the genes, using oligonucleotide complexes for the phenotypic and/or genotypic restoration of defective genes. Approaches to an oligonucleotide–based strategy to achieve this goal have been tested. Chimeric RNA/DNA oligonucleotides and single-stranded

oligonucleotides were developed for site-specific correction of episomal and chromosomal target genes [see Andersen et al. J Mol Med. (2002) 80, 770-81; Alexeev et al., Gene therapy (2002) 9, 1667-1675; Kmiec, Clin. Invest. (2003) 112, 632-636; Wu et al., J Biomed Sci. (2001) 8, 439-45; Yoon, PA # US1999000473872; Davis et al., PA # US1000767775; Youn el al., PA # USUS2001000962628; Kmiec et al., PA # US2002000260375; Kmiec et al., PA # US2002000215432]. Experiments demonstrated the feasibility of using chimeric RNA/DNA and single stranded oligonucleotides to introduce point conversions in genes *in vitro* and *in vivo*. This gene repair approach relies on hybridization of the chimera to the target gene, generating a mismatch with the targeted point mutation. Restored gene function was anticipated to occur through activation of endogenous repair systems that recognize the created mismatch [see Andersen et al., J Mol Med. (2002) 80, 770-81; Alexeev et al., Gene therapy (2002) 9, 1667-1675; Kmiec, Clin. Invest. (2003) 112, 632-636; Wu et al., J Biomed Sci. (2001) 8, 439-45; Wang et al., (2003) Proc. Natl. Acad. Sci. USA 100, 14822-14827]. Double stranded oligonucleotides have been tested for site specific gene alteration in plant cells [see Arntzen et al., PA # US1998000129298; Kmiec, AP # US1994000353657].

Triplex forming oligonucleotides also have been employed as sequence-specific tools for gene targeting. Triplex forming oligonucleotides bind in the major groove of double stranded DNA, with high affinity. Because of this characteristic, triplex forming oligonucleotides have been proposed as tools for the site specific corrections of targeted genes [see Knauert et al., Hum Mol Genet. (2001) 10, 2243-2251; Richardson et al., Drug Target (2002) 10, 133-134; Thoung et al., (1993) Angewandte Chemie. Intl. Ed. Eng., 32, 666-690.].

Current targeted gene repair methods are controversial and still at the level of development, however only a very low level of repair has been found. There is a need for more effective tools, in order to obtain phenotypic or genotypic restoration of defective genes in somatic tissues.

Summary

The invention features a method for targeted gene repair by RNA editing. Any genetic defect, e.g., a substititution, deletion, or addition of a basepair, compared to a normal wild type sequence is phenotypically changed using the method described above. The method is carried

out by contacting a genomic target DNA of a cell with a hybrid RNA oligonucleotide complex, which contains a first strand and a second strand. The first and second strand are annealed to one another to form the complex. The first strand contains a flanking sequence that is complementary to sequence of the target DNA ("sticky ends") and a central sequence (located between the flanking sequence) that contains at least one non-complementary nucleotide in a location opposite a defect of the target DNA. The central sequence, which is RNase-sensitive, is at least a triplet, and is preferably at least 4, 5, 6, or more nucleotides in length. The flanking sequence of the first strand includes an RNase H-resistant modification, e.g., addition of a 2-O-methyl moiety, to both a 3' and 5' to the non-complemenary nucleotide. The second strand of the complex is shorter than first strand of the complex, and the second and first strands are complementary to and annealed to one another. The complex is allowed to hybridize to target genomic DNA of the target cell, and a repaired RNA is produced in the cell. The repaired RNA contains a sequence alteration opposite the defect of the target DNA, and the genomic target DNA sequence remains unaltered. The RNA alteration or phenotypic change accomplished by the method is not maintained in progeny of the cell.

The flanking sequence of the first strand contains at least four complementary nucleotides (relative to the genomic target DNA) 3' to the non-complementary base pair (of the target DNA and first strand RNA oligonucletide) and at least four complementary nucleotides (relativet to the genomic target DNA) 5' to non-complementary base pair. The flanking portion of the sequence contains a RNase H-resistant modification, whereas the central portion of the sequence (opposite the defect) does not contain such a modification and is therefore nuclease and Rnase sensitive, i.e., the nucleotide bonds in the central region are enzymatically cleaved.

The first strand optionally contains one or more phosphorothioate linkages and is at least 15 nucleotides in length. For example, the first strand contains at least 16 18, 20, 30, 40, 50 or more nucletoides, the flanking sequence of which contains at least 2 nucleotides that are complementary to target genomic DNA in the region of the defect. The complementary flanking sequence on either side of the defect is 5, 10, 15, 20, 25 or more nucleotides in length. The second strand of the oligonucleotide complex is at least 7 nucleotides in length and is optionally longer, e.g., 9, 12, 15, 20 nucleotides.

Further featured is a method of modifying a cell phenotype by altering expression of a target gene, by contacting the cell with an oligonucleotide complex comprising a first strand and

a second strand, the first strand having a nucleotide sequence substantially complementary to an RNA expressed from the target gene, the oligonucleotide having a central and flanking segments, the central segment and the nucleotide sequence of the target gene being non-complementary in at least one nucleotide, and the flanking segments having at least one chemical modification or derivative such that at least one flanking segment is more nuclease resistant than an otherwise identical unmodified oligonucleotide having the same sequence; and a second strand having a nucleotide sequence substantially the same as the RNA expressed from the target gene and is complementary to at least a portion of the central segment of first strand, wherein the complementary portion comprises the central segment of the first strand, the first strand and second strand forming the complex by base pairing in at least the central segment, the complex having at least one single-stranded flanking segment; and measuring the phenotype of the cell with respect to the target gene, wherein an altered phenotype for the gene indicates that the central segment has altered expression of the target gene. Accordingly, the nucleotide modifications are selected from the group 2'-O-methyl ribosyl; phosphorothioate; peptide nucleic acid; 2'-halo, 2'-fluoro, 2'-alkyl, 2'-alkoxylalkyl, bridged sugars and similar chemical moieties; methylphosphonate, ethylphosphonate, phosphoramidate, thiophosphate, dithiophosphate, morpholino, boronophosphate, morpholinophosphate internucleoside linkages and similar chemical moieties; derivatized heterocycles, and sugars. Further, the cell is from a patient having a genetic disease. In some embodiments, the cell is an isolated cell in a culture. The method can further involve isolating and sequencing RNA from a contacted cell having a different phenotype from cells from the patient or the culture, wherein altering expression of the target gene is altering an RNA nucleotide sequence.

In general in these methods, the target gene carries a mutation. The central segment of the complex has a normal sequence compared to the target gene. Further, the modification confers ribonuclease resistance. Further, the modification confers induction of ribonuclease activity in the cell. In general, the central segment is at least about 4 nucleotides in length, at least about 6 nucleotides in length. Further, the first strand is at least about 17 nucleotides in length, and the second strand is at least about 17 nucleotides in length. In a specific embodiment of the method, at least one nucleotide is a 2'-O-methyl ribosyl nucleotide. For example, there is a plurality of 2'-O-methyl ribosyl nucleotides. The second strand can further contain a small chemical group at a 5' or 3' terminus, or the second strand further contains a small chemical

group at both 5' and 3' termini. Further, the modification can be located at a nucleotide in a 3' or a 5' terminus. The second strand further comprises a modification comprising a small chemical group in a 3' or a 5' terminus. For example, the small chemical group is selected from phosphate, diphosphate, triphosphate, thiophosphate, dithiophosphate, aldehyde, carboxy, dihydroxy, hydroxyl, methyl, ethyl, sulfhdryl, sulfate, and boronate. Further in a related embodiment, the central segment comprises at least one modification which is a phosphorothioate.

In general, the genetic disease is selected from the group of albinism, cystic fibrosis, muscular dystrophy, myotonic dystrophy, muscular atrophy, sickle cell anemia, hepatic disorder, hemophilia, Crigler-Najjar syndrome, renal tubular acidosis, β-thalassemia, atherosclerosis, Huntington's disease, spinocerebellar ataxia, Machado-Joseph disease, Fragile X, and Frederich's ataxia.

In another embodiment, the invention provides an oligonucleotide complex comprising a first strand and a second strand, the first strand having a nucleotide sequence substantially complementary to an RNA expressed from a target gene, the oligonucleotide having a central and flanking segments, the central segment and the nucleotide sequence of the target gene being non-complementary in at least one nucleotide, and the flanking segments having at least one chemical modification or derivative such that at least one flanking segment is more nuclease resistant than an otherwise identical unmodified oligonucleotide having the same sequence; and a second strand having a nucleotide sequence substantially the same as the RNA expressed from the target gene and which is complementary to at least a portion of the central segment of first strand, wherein the complementary portion comprises the central segment of the first strand, the first strand and second strand forming the complex by base pairing in at least the central segment, the complex having at least one single-stranded flanking segment;

The oligonucleotide complex technique provided herein suggests a new general approach to modulation of genetic diseases, including but not limited to those diseases mentioned above. The oligonucleotide complex technique provides functional restoration of the mutated genes by deletion/insertion of the segment of RNA (or DNA). The oligonucleotide complex technique method inserts one or more deleted nucleotide(s) into the targeted region of specific RNA (or DNA). The oligonucleotide complex technique facilitates simultaneous correction of more then

one mutation with a single set of oligonucleotide complex into a single targeted RNA (or DNA) gene.

Site-specific insertion of the specific RNA (or DNA) genes with the oligonucleotide complex technique may require one or more treatment steps with the oligonucleotide complex. Site-specific alteration of gene with the oligonucleotide complex technique may be performed in one or more targeted genes by using several oligonucleotide complex sets consecutively as required, or even simultaneously. For enhancing targeted gene repair efficiency using the oligonucleotide complex technique this complex is used synergistically with other known or potential therapeutic compounds. The oligonucleotide complex technique is used for targeting one or several harmful genes one by one or simultaneously. The oligonucleotide complex technique is used for extension of translation of targeted genes (by elimination of undesirable stop codons). The oligonucleotide complex technique is used for creation of "new genes" by insertion of transcription or translation sites in certain regions of the mammalian genome

The oligonucleotide complex technique is used for deletion or insertion of purine or pyrimidine repeats at the ends of different genomic RNA or DNAs. The oligonucleotide complex technique is used for the deletion or diminution of a region of RNA containing a reiteration of excess triplets. The oligonucleotide complex technique provides insertion of UGU in a sufficient fraction of Δ508 mRNA to induce phenotypic reversion in a tissue culture cell line. The oligonucleotide complex technique according of SPQ data indicates suppression of the chloride anion conductance, or in the other words phenotypic restoration of a mutated Δ508 gene. The oligonucleotide complex technique according to subcloning data presented herein shows approximately 30 percent insertion of UGU in the site of the Δ508 mRNA UUU triplet deletion. The oligonucleotide complex technique is used for deletion or insertion of purine or pyrimidine repeats at the ends of specific genomic RNA or DNAs. The oligonucleotide complex technique inserts UGU in the site of the Δ508 mRNA UUU triplet deletion with one step addition of the oligonucleotide deletion/insertion hybridized RNA/modified RNA duplex. The oligonucleotide complex technique inserts UGU in the site of the Δ508 mRNA UUU triplet deletion with more than one (two) step treatment. The first step treatment was performed with RNase H (or other endonucleolytic enzyme) deleting single strand RNA. The second step of the treatment was performed by the oligonucleotide deletion/insertion RNA/modified RNA duplex. The oligonucleotide complex technique may require more then one step treatment (i.e.

consecutive steps) for the restoration of targeted RNA (DNA) gene(s). RNase H (or other endonucleolytic enzyme) deletes a specific region of RNA, plus the pre-hybridized duplex of oligonucleotides involved in the oligonucleotide complex technique include, but are not limited to, standard oligonucleotides, modified oligonucleotides, standard and modified oligonucleotides with different sequential alteration, standard or modified oligonucleotide in the middle and flanked by different derivatives of oligonucleotides, derivatives of oligonucleotides facilitating different endonucleolytic deletions and/or some other "gene insertion/deletion" catalysis, oligonucleotides conjugated with different chemical groups (such as, but not limited to, intercalators, groove binders, alkylating reagents, photoactive groups (such as psoraren) and other moieties) .

Compositions of oligonucleotide derivatives in the oligonucleotide complex technique include, but are not limited to standard and modified oligonucleotides with different sequential alteration. Oligonucleotide derivatives in the oligonucleotide complex technique may contain small chemical groups at the 5'- and /or 3'-end, including but not limited to, phosphate, diphosphate, triphosphate, thiophosphate, dithiophosphate, aldehyde, carboxyl, dihydroxyl, hydroxyl, methyl, ethyl, sulfhydryl, sulfate, Boronates, and similar chemical moieties. Oligonucleotide derivatives in the oligonucleotide complex technique may include, but are not limited to PNA; 2'-halo, 2'-fluoro, 2'-alkil, 2'-alkoxylalkyl, bridged sugars and similar chemical moieties; methylphosphonate, ethylphosphonate, phosphoramidate, thiophosphate, dithiophosphate, morpholino, Boronophosphate, morpholinophosphate internucleoside linkages and similar chemical moieties; oligonucleotides with derivatized heterocycles, sugars and/or internucleoside linkages and similar chemical moieties. Oligonucleotide derivatives in the oligonucleotide complex technique may include, but are not limited only to derivatized heterocycles, sugars or internucleoside linkages, but also to combination of these moieties mentioned above.

RNase H- (or other endonucleolytic enzyme-) activating single stranded oligonucleotide and pre-hybridized duplex of oligonucleotides involved in the oligonucleotide complex technique method may be four or more (up to three hundred) base long. The deletion/insertion oligonucleotide duplex used in the oligonucleotide complex technique method maybe composed of oligonucleotides of different length. The deletion/insertion oligonucleotide duplex employed in the oligonucleotide complex technique may possess the 5'- and/or 3'- "sticky ends". The

length of "the sticky ends" in the deletion/insertion oligonucleotide duplex employed in the oligonucleotide complex technique may be one base and more (up to 40-60 bases). The double strand part of the deletion/insertion oligonucleotide duplex employed in the oligonucleotide complex technique may be 4-5 base pairs or more (up to 100 base pairs).

Sources of the derivatives of oligonucleotide used in the oligonucleotide complex technique method are chemically or biologically synthesized. The oligonucleotide complex technique is also useful to target with an oligonucleotide sequence specifically the deletion or insertion of a portion of a gene from a pathogenic bacterium, virus, insect, arthropod, parasite, land or marine plant or other living organism; to silence a virulence factor, thereby rendering that organism into a non-pathogenic one. This technique is used for preparation of a vaccine against the pathogen, or a therapeutic treatment by spray or other means of obtaining entry to the pathogen.

The oligonucleotide complex technique is used for the suppression/inactivation of biological pathogens in body fluids either *in vitro* or *in vivo*. The oligonucleotide complex technique is used for diagnostic analytical purposes or as a tool for a laboratory research.

Brief Descriptions of the Drawings

Scheme 1 is a drawing of (A) a hypothetical scheme for repair of CFTR Δ508 mRNA by CF4/CF6 duplex, and (B) sequences found in restored mRNA. Bold italic type corresponds to bases of 2'-O-methyl ribosyl oligonucleotides with normal internucleoside phosphate bonds (CF4 oligonucleotide). Regular type corresponds to natural RNA. Bold plus underlined type corresponds to inserted bases of ribonucleotides. Shaded bases in the CF4 and Δ508 mRNA indicate non-complementary Watson-Crick base pairs. Sequence analysis of RT-PCR products obtained by using different primers is also shown. (a) A in square indicates that in 7 out of 10 sequencings this A was present, but in 3 other sequencings was absent, leaving 3 deletions. (b) Dashes indicate deletions, possibly induced by PCR. (c) There are no deletions. U in square indicates that in this position U sometimes is replaced by another nucleotide.

Fig. 1. is a set of tracings of whole cell currents of Δ508CFTR expressing cells. Top, Representative tracings of Δ508CFTR expressing cells after treatment with CF4/CF6 were

obtained before and after stimulation with a cAMP mixture. The cAMP-activated currents were inhibited by DPC (500 μM). Bottom, Left: untreated Δ508 cells lacked a cAMP activated whole cell conductance (n=21). Center: In contrast, CF4/CF6 treated Δ508 cells had a robust cAMP response (Linear whole-cell currents were observed before and after cAMP activation). Right: cAMP activation in treated cells was inhibited by DPC. Results essentially similar to those shown were obtained when the initially used two step insertion mechanism was employed.

Fig. 2. is a set of tracings of single channel currents of Δ508CFTR expressing cells. A. Top: representative tracing of current observed in Δ508CFTR expressing cells after treatment with CF4/CF6. Currents were obtained in excised inside-out patches. A. Bottom: addition of PKA (100 nM) and MgATP (1 mM) induced a rapid channel activation. Data are representative of n=12 experiments. No activation was observed in control Δ508CFTR expressing cells (data not shown). B. Top: the PKA-activated Cl^- single channel currents had a single channel conductance of 12 pS (n=6). B. Bottom: details of Cl^- single channel conductance are shown in the all-point histograms.

Fig. 3. is a set of bar graphs that show the results of an SPQ fluorescence assay. Cl^- induced changes in fluorescence were followed in cells loaded with the Cl^- sensitive dye SPQ. SPQ fluorescence was tested in WT1 cells expressing wild type CFTR, and control and treated Δ508 cells in a custom-made chamber, under UV-fluorescence microscopy. A cAMP-induced response (5-15 min) was only observed in WT1 and treated Δ508 cells ($p<0.05$). Numbers in parentheses indicate individual cells analyzed.

Fig. 4. is a photograph of an agarose gel electrophoretogram showing allele-specific RT-PCR analysis of CFTR mRNA. Samples from WT1 mRNA were diluted into Δ508 mRNA (lanes 2 to 14, from undiluted to 10^6 order with 10-fold serial dilutions) to test efficiency of allele-specific primers (CFFW and CFFM, Table 1). Lanes 2 and 3 show WT1 and Δ508 mRNA amplified with respective primers. The wild type primers recognized WT1 mRNA in dilutions of up to 1: 10^5 (Lanes 11 and 12). Gel electrophoresis was conducted in agarose gels (3%), and a 25 base pair (bp) ladder is shown on the left lane.

Fig. 5. is a photograph of an agarose gel electrophoretogram showing specific reverse primer analysis of mRNA in samples of RT-PCR material obtained by amplification with wild type (N1) and mutant (M1) specific reverse primers (Table 1). Lanes 1 and 2 are Δ508 untreated and RT PCR with N1 and M1 primers respectively. Similarly lanes 3 and 4 are Δ508 treated with CF4/CF6 and following RT PCR with N1 and M1 primers respectively. Gel electrophoresis was performed in 3% agarose gel.

Fig. 6. is an automated tracing by type of nucleotide base obtained from DNA sequencing of RT-PCR products. Sequence analysis of the CF4/CF6 treated Δ508CFTR was conducted by RT-PCR analysis of total RNA. The PCR materials observed in the gel were subcloned in pCR-Blunt vector and sequenced at the MGH Sequencing Facility. Minor peaks represent background. Variations in heights of peaks are due to instrumental sensitivity

Scheme 2 is a drawing of a hypothetical site-specific deletion/insertion mechanism in the RNA containing mismatch region, with the two step nucleotide complex technique. Red corresponds to targeted nucleotide(s), black and white correspond to hybrid oligonucleotide for RNase-H activation, yellow corresponds to natural RNA , grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/repaired segment of RNA

Scheme 3 is a drawing of a hypothetical site-specific deletion/insertion mechanism in the RNA containing mismatch region, with the one step oligonucleotide complex technique. Red corresponds to targeted nucleotide(s), yellow corresponds to natural RNA, grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/repaired segment of RNA

Scheme 4 is a drawing of a hypothetical site-specific deletion/insertion mechanism in the RNA containing deleted region, with the two step oligonucleotide complex technique. Black and white correspond to hybrid oligonucleotide for RNase-H activation, yellow corresponds to natural RNA , grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/repaired segment of RNA

Scheme 5 is a drawing of a hypothetical site-specific insertion/deletion mechanism in the RNA containing deleted region, with the one step oligonucleotide complex technique. Yellow corresponds to natural RNA, grey corresponds to template oligonucleotide in "insertion duplex", and blue corresponds to inserted/repaired segment of RNA (or DNA).

Scheme 6 is a drawing of a hypothetical site-specific deletion/insertion mechanism in the RNA containing repeat mutations, with the two step oligonucleotide complex technique. Red corresponds to targeted nucleotide(s), black and white correspond to hybrid oligonucleotide for RNase-H activation, yellow corresponds to natural RNA, grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/repaired segment of RNA

Scheme 7 is a drawing of a hypothetical site-specific deletion/insertion mechanism in the RNA containing repeat mutations, with the one step oligonucleotide complex technique. Red corresponds to targeted nucleotide(s), yellow corresponds to natural RNA, grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/repaired segment of RNA. The XXX indicates the size of the base of the oligonucleotide loop. It may vary between zero and twenty.

Scheme 8 is a drawing of a hypothetical site-specific frame changing mechanism in the active RNA, with the two step oligonucleotide complex technique. Red corresponds to targeted nucleotide(s), black and white correspond to hybrid oligonucleotide for RNase-H activation, yellow corresponds to natural RNA , grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/frame changed segment of RNA

Scheme 9 is a drawing of a hypothetical site-specific frame changing mechanism in the active RNA, with the one step oligonucleotide complex technique. Red corresponds to targeted nucleotide(s), yellow corresponds to natural RNA, grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/frame changed segment of RNA.

Scheme 10 is a drawing of a hypothetical site-specific transcription or translation start site insertion mechanism in the active RNA, with the two step oligonucleotide complex technique. Red corresponds to targeted nucleotide(s), black and white correspond to hybrid oligonucleotide for RNase-H activation, yellow corresponds to natural RNA , grey corresponds to template oligonucleotide in insertion/deletion duplex, and blue corresponds to inserted/frame changed segment of RNA.

Scheme 11 is a drawing of a hypothetical scheme for repair of Inactive tyrosinase by two step oligonucleotide complex technique. Bold italic type corresponds to natural RNA. Small caps correspond to PS ODN. Shaded corresponds 2'-o-methylribosyl oligonucleotides, with internucleoside phosphate bonds. Underline base corresponds targeted base. Base in squire correspond replaced base.

Scheme 12 is a drawing of a hypothetical scheme for repair of Inactive tyrosinase by one step oligonucleotide complex technique. Bold italic type corresponds to natural RNA. Small caps correspond to thiophosphate oligodeoxynucleotide. Shaded corresponds 2'-o-methylribosyl oligonucleotides, with internucleoside phosphate bonds. Underline base corresponds targeted base. Base in squire correspond replaced base.

Scheme 13 is a drawing of a hypothetical scheme for repair of Cystic Fibrosis Δ508 mRNA by two step oligonucleotide complex technique. Bold italic type corresponds to natural RNA. Small caps correspond to thiophosphate oligodeoxynucleotyde. Shaded corresponds 2'-o-methylribosyl oligonucleotides, with internucleoside phosphate bonds. * and _ _ _ corresponds deletion region of mutated mRNA.

Scheme 14 is a drawing of a hypothetical scheme for repair of Cystic Fibrosis Δ508 mRNA by one step oligonucleotide complex technique. Bold italic type corresponds to natural RNA. Shaded corresponds 2'-o-methylribosyl oligonucleotides, with internucleoside phosphate bonds. * and _ _ _ correspond to a deletion region of mutated mRNA.

Detailed Description

Data herein show oligonucleotide complex compounds and methods for the TGR and restoration of a cystic fibrosis phenotype in a cultured Δ508CFTR cell line, using hybrid oligonucleotides and a special modified chimera of an RNA/RNA (DNA) duplex.

Cystic Fibrosis (CF) is a lethal disorder caused by mutations in the CFTR gene encoding the CFTR channel [Riordan, et al., J.R., (1989) Science, 245, 1066-1073; Kerem et al., (1989) Science, 245, 1073-1080]; Burke (2003), Eng. J. Med. 349, 969-974]. CFTR, a cAMP- activated anion channel [Anderson et al., (1991) Science, 251, 679-682; Bear et al., (1992) Cell, 68, 809-818], is associated with the dysfunctionality of epithelia in several tissues [Crawford et al., (1991) Proc. Natl. Acad. Sci. USA 88, 9262-9266] including lungs, pancreas, intestine, sweat glands and kidneys. CF is the most common lethal genetic disease of Caucasians, affecting 1 in 2000 individuals. More than 150 mutations have been identified in the CFTR gene [Tsui, (1992) Trends Genet. 8, 391-398], associated with a pleiotropic spectrum of CF phenotypes [Cutting, (1993), J. Bioenerg. Biomembr. 25, 7-10; Strandvik et al., (2001), Genet. Test. 5, 235-242]. New mutations have been found with distinctive impacts on CF populations [Gilfillan et al., (1998), J. Med. Genet. 35, 122-125; Onay et al., (1998), Hum. Genet. 102, 224-230; Visich et al., (2002), Clin. Genet. 61, 207-213]. However, the most frequent CFTR mutation, accounting for almost 75% of all cases of the disease, is a deletion of three bases (TTT), encoding the amino acid phenylalanine in position 508 of the translation sequence, accompanied by C to U replacement immediately 5' to the deletion. This deletion is often accompanied by a mismatch immediately adjacent in the 5' direction to the deletion: in a U in place of a C.

The Δ508 CFTR is a misfolded but partially functional channel protein [Li et al., (1993) Nature Genet. 3, 311-316; Pasyk et al., (1995) J. Biol. Chem. 270, 12347-12350], unable to translocate perfectly to target plasma membranes [Denning et al., (1992) Nature, 358, 761-764; Cheng at al., (1990), Cell, 63, 827-834]. Several chemical and pharmacological strategies have been attempted, to rescue the CF phenotype at the cellular level, to bring Δ508 CFTR to the plasma membrane. Partial success has also been achieved by adenoviral vector infection [Boucher et al., (1994), Hum. Gene Ther. 5, 615-639; Teramoto et al., (1998), J. Virol. 72, 8904-8912] and re-insertion of the wild type CFTR gene into a CF genetic background. More recently, a novel repair strategy was used, based on trans-splicing of the Δ508CFTR pre-mRNA [Liu at al., (2002), Biotechnol. 20, 47-52; Puttaraju et al., (2001), Mol. Ther. 4, 105-114; Mansfield et al., (2000) Gene Ther. 7, 1885-1895]. Gentamycin has also been found to induce a

correction of faulty CFTR function in CF, caused by CFTR stop mutations [Bedwell et al., (1997) Nature Med., 3, 1280-1284], rather than by the TTT deletion.

Loss or suppression of disease-associated gene function by antisense oligonucleotide technologies involves specific inhibition of DNA, RNA and protein expression [Zamecnik et al., (1978), Proc. Natl. Acad. Sci. USA 74, 280-284; Stephenson et al., (1978) Proc. Natl. Acad. Sci. USA 75, 285-288]. This is based in good part on complementary hybridization of synthetic oligonucleotides with the natural sequences in either DNA or RNA [Temsamani et al., (1994) Antisense Res. Devel. 4, 279-284]. Triplex-forming oligonucleotides have also been used for the modification of cellular gene function [Felsenfeld et al., (1957) J. Am. Chem. Soc., 79, 2023-2024; Thoung et al., (1993) Angewandte Chemie. Intl. Ed. Eng., 32, 666-690; Agrawal et al., (1990). Proc. Natl. Acad. Sci. USA 87, 1401-1405]. The consequence of such interaction is a competitive blockade of either DNA or RNA synthesis at replication or transcription, respectively and, in the case of exons, at the translational steps in protein synthesis. Upon cell entry, exogenous complementary deoxyoligonucleotides hybridize with a target mRNA, inducing an excision endonucleolytic effect on the mRNA, a so-called ribonuclease H (RNAse H) effect . resulting in synergism with hybridization inhibition [see Zamecnik et al., (1996) Ed. By Sudhir Agrawal, Humana Press, NJ, 1-11]. Likewise, RNA editing [Simpson et al., (1996) Annu. Rev. Neurosci. 19, 27-52] has been reported in numerous cell systems where nucleotide sequences can also be modified at the RNA level. RNA editing encompasses various mechanisms, including base substitutions and deletions [Simpson et al., (1996) Annu. Rev. Neurosci. 19, 27-52]. RNA duplexes with internal and external guide sequences in some cases are required to drive these reactions.

Double stranded RNA sequences have a role in RNA interference (RNAi) and gene silencing at the transcriptional level [Hannon, (2002) Nature, 418, 244-251]. RNAi mediated gene silencing was discovered in *C. elegans* [Fire et al., (1998), Nature, 391, 806-811; Montgomery et al., (1998), Proc. Natl. Acad. Sci. USA 95, 15502-15507; Grishok et al., (2000), Science, 287, 2494-2497], and has been observed in numerous cell models and organisms [Hannon, (2002) Nature, 418, 244-251; Hammond et al., (2000), Nature, 404, 293-296; Silva at al., (2002), Trends Mol. Med. 8, 505-508. Gene silencing results from successive cleavage of long dsRNA, particularly originating from viruses, to oligonucleotide siRNAs by DICER enzymes [Zamore et al., (2000) Cell, 101, 25-33; Bernstein et al., (2001), Nature, 409, 363-366].

After oligonucleotide hybridization of siRNA with mRNA, the cleavage of target mRNA is catalyzed [Hammond et al., (2000), Nature, 404, 293-296; Zamore et al., (2000) Cell, 101, 25-33]. RNAi phenomena have been used for target gene silencing from nematodes and plants to mammals [Hannon, (2002) Nature, 418, 244-251]. Separate from the above is CpG stimulation of host immunomodulatory mechanisms, in both prokaryotic and eukaryotic organisms [Krieg (2003) Nat. Med., 9, 831-835; Kandimalla et al., (2003) Biochemical Society Transactions, 31, 654-658].

The gain of function and/or correction of defective genes present a continuing challenge for gene therapy. Partial restoration of function in the Δ508 CFTR background has been shown to occur by membrane insertion of Δ508 CFTR without changes in either phenotype or genotype [Brown et al., (1996) Cell Stress & Chaperones, 1, 117-125; Arispe et al., (1998) J. Biol. Chem. 273, 5727-3574]. This is based on the capacity of Δ508 CFTR to function even as a misfolded protein [Welsh et al., (1993), Cell, 73, 1251-1254; Ward et al., (1994), J. Biol. Chem. 269, 25710-25718]. Its translocation to a plasma membrane may incompletely restore functionality of the phenotype RNA/DNA oligonucleotide hybrids [Cole-Strauss et al., (1996), Science, 273, 1386-1389; Yoon at al., (1996), Proc. Natl. Acad. Sci. USA 93, 2071-2076; Alexeev et al., (1998), Nature Biotech. 15, 1343-1346; Parekh-Olmedo et al., (2001), Sci. STKE 73, PL1] and single stranded oligonucleotides [Igoucheva et al., (2001) Gene Therapy, 8, 391-399] have been used for the correction of defective genes. Substituted, circular, single stranded RNA/DNA chimeras have been employed to insert base pairs in deficient genomic DNA [Cole-Strauss et al., (1996), Science, 273, 1386-1389; Yoon at al., (1996), Proc. Natl. Acad. Sci. USA 93, 2071-2076]. In these studies, nucleotide exchange of target episomic and genomic DNA was reported using chimeric RNA/DNA oligonucleotides. Some attempts at replicating this work, however, showed no nucleotide exchange in the targeted loci by cloning of the PCR products [Zhang et al., (1998) Antisense & Nucleic Acid Drug Developm. 8, 531-536]. Results were viewed by these and other investigators as PCR artifacts created by the RNA/DNA oligonucleotides themselves [Zhang et al., (1998) Antisense & Nucleic Acid Drug Developm. 8, 531-536; Taubes, (2002) Science, 298, 2116-2120]. Transcriptional repair has been used for repair of mRNA. Spliceosome mediated cis-splicing of pre-mRNA is an essential step in gene expression [Jurica et al., (2003), Mol. Cell, 12, 5-14]. Other trans-splicing mechanisms in pre-mRNA molecules have been shown to form functional hybrid mRNA molecules in different mammalian systems

[Spector, (1993), Curr. Opin. Cell Biol. 5, 442-447; Harris et al., (1990), Nucleic Acids Res. 18, 3015-3019]. Spliceosome mediated RNA transplicing (SmaRT) technology has also been used to modify Δ508 CFTR transcripts in human CF airway epithelia [**Liu at al.,** (2002), Biotechnol. 20, 47-52].

An mRNA hybridized to a single stranded synthetic short piece of DNA was used to activate RNase H [Agrawal et al., (1990). Proc. Natl. Acad. Sci. USA 87, 1401-1405], which then specifically hydrolyzed only the hybridized segment of mRNA. RNase H as an ubiquitous enzyme had been previously known [[Agrawal et al., (1990). Proc. Natl. Acad. Sci. USA 87, 1401-1405]]. This work, and others define nucleotide limits of RNase H activity in a precise way. At least a 4-6 nucleotide internally located region in an mRNA, which is hybridized to a phosphorothioate modified (PS) oligodeoxyribonucleotide, is needed for Rnase-mediated excision. If a precise small segment of mRNA, which included the deleted UUU region (the ribosomal substitute for TTT) were synthesized and annealed to the larger chimera, which induced/activated a small deletion in mRNA, an insertion of the somewhat larger total piece excised might restore the phenotypic effect at the mRNA level.

Initially, a modified oligodeoxyribonucleotide with a central segment containing a phosphorothioate (PS) modification was constructed, with flanking segments PS plus 2'-O-methyl modifications. This was designed to hybridize in Watson-Crick base complementarity to the region of Δ508 mRNA where the PS section would be directly opposite the Δ508 mRNA position, flanked 3'- and 5'- by an adjacent few nucleotides complementary to the Δ508 mRNA. In this way the PS segment would activate endogenous RNase H, and cleave those bases opposite the PS complementary oligonucleotide. The 2'-O-methyl PS, plus segments on both sides of the RNase H sensitive section would be generally nuclease and RNase H resistant and would serve in this way as a "genetic band aid". Without being limited by any particular mechanism, the flanking segments are intended to hold the 5'- and 3'- segments of Δ508 mRNA in place, in position for a possible insertion or repair of bases in a second step. For the possible repair step two single complementary strand oligonucleotides were constructed: Cystic Fibrosis 4 (CF4) and Cystic Fibrosis 6 (CF6), as shown in Scheme 1. This combination was annealed to form a duplex. The duplex was added to growing Δ508 CFTR cell cultures. Patch clamp examination [Reisin et al., (1994) J. Biol. Chem. 269, 20584-20591] showed evidence of phenotypic reversion (Figures 1 and 2). When the CF4/CF6 annealed duplex without

oligonucleotide treatment, as described above, was, however, added to the tissue culture cells, phenotypic reversion was likewise found, based on the patch clamp technique. A one step phenotypic reversion technique was chosen, as a simpler model for the sequencing studies described below.

CF4 and CF6 were annealed to form an oligonucleotide duplex (CF4/CF6) (Table 1) and were then added to the Δ508 cells in tissue culture. There was restoration of the CFTR channel to functional normality, as determined by patch clamping and 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) quenching techniques. Under those conditions, restoration of function was found to occur in 8-18 hours (Figures 1 and 2). As 2'-O-methyl modified CF4 has been reported not to activate RNase H [Shen et al., (1998) Bioorg. Med. Chem., 6, 1695-1705], there would appear to be another endonucleolytic enzyme responsible for the base excision, unless RNase H is activated by a triple-stranded oligonucleotide, in which the complementary 2'-O-methyl moiety is present.

Under Rose experimental conditions there was complete restoration of CFTR function, whereas non-insertion reversions have been only partial. [Rose D M et al, Eur J Res 2000;5, 9-12] On replication of the human tissue culture cells and washing out of the oligonucleotide after a few days incubation, the restoration of CFTR function was lost. This strongly suggested a phenotypic but not genotypic (i.e. RNA but not DNA) restoration of functionality of the CFTR channel. Amplification and sequencing technology was used to determine whether trinucleotide insertion into Δ508 mRNA has been made. Scheme 1 is a schematic diagram of the proposed insertional mechanism.

An oligonucleotide complex (CF4/CF6, respectively 2'-0-methyl RNA/unmodified RNA oligonucleotide duplex) was used herein to restore CFTR function by insertion of missing bases in Δ508CFTR mRNA from a cultured (Δ508) cell line. Cyclic AMP-activated (cAMP) whole cell currents and Cl^- transport were detected in CF4/CF6 treated, rather than control Δ508 cells by the patch clamping and by SPQ fluorescence quenching analysis, respectively. Further, the nucleotide addition in the deleted region of Δ508 CFTR was determined after amplification by the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Insertion of UGU and replacement of U by C immediately 5' to the deletion site in Δ508 mRNA appear to have taken place, with phenotypic but not genotypic reversion in tissue culture of treated cells. The mechanism of insertion of nucleotides is yet to be determined.

The examples provided herein demonstrate feasibility of using an oligonucleotide complex technique as a therapeutic and/or prophylactic agent for the phenotypic and restoration of function of a mutated gene. The invention having now been fully described, it is exemplified by the following examples and claims, which are not to be construed as further limiting. The contents of all references cited are incorporated in their entireties herein by reference.

EXAMPLES

Oligonucleotide complexes and methods for their use for the functional restoration of the mutated genes by deletion/insertion mechanism of the segment of RNA are provided.

Several genetic diseases relate to disorders in the sequences of mutated genes and genes with inborn errors. Other major sources of genetic diseases, shown above, are due to "switch on" silent genes or presence of viral genes in the mammalian genome.

The cause of a fatal or serious genetic disease may be as small as a single mismatch in base pair (one mismatch, one inserted/deleted base pair in the whole gene sequence) or absence of a partial or whole gene in the genome. Consequently, availability of the highly specific oligonucleotide complex technique for the restoration of the targeted (defected) gene could be a powerful tool to combat currently incurable genetic diseases.

Site-specific insertion of the genes with the oligonucleotide complex technique (as shown herein as replacement of a cystic fibrosis phenotype in a cultured Δ508CFTR cell line experiments) can occur with a one or two step oligonucleotide complex treatment. Below are shown schemes for restoration of mutated and inborn error genes (Schemes 2-7), and schemes of switch-on and/or switch-off targeted genes (Schemes 8-10) using an oligonucleotide complex technique.

One step and two step oligonucleotide complex treatment of targeted RNA (or DNA) are shown in the Schemes 3, 5, 7, 9 and 2, 4, 6, 8, 10 respectively.

In Scheme 2 is shown a hypothetical site-specific deletion/insertion mechanism at the targeted region of RNA with the two step oligonucleotide complex technique. The targeted region in this case could be one (or a few) mismatched bases or one (or a few) inserted bases in the RNA sequence. On the first step, oligonucleotide for RNase-H activation deletes the target

region of RNA, and on the second step insertion of the desirable sequence takes place. The RNase H deleted region could be a few or more bases, depending on the extend of RNase induced deletion, as a result of which the RNA oligonucleotide from the insertion duplex might be spliced into the deleted region, or alternatively serve as a triplex backbone sequence for a one-by-one insertion mechanism.

In Scheme 3 is shown site-specific deletion/insertion mechanism of the targeted region of RNA with the one step oligonucleotide complex technique. There are different possibilities of one step restoration: triplex backbone sequence formation by a one-by-one insertion mechanism, with specific docking and cleavage of selective nucleotide sequences which are hybridized to an RNA (or DNA) sequence. The cleavage might by induced by either a new enzyme or the Dicer enzyme, and as a result, production of RNA (or DNA) with a corrected (inserted) region.

In Scheme 4 is shown site-specific insertion mechanism of the deleted region of RNA with the two step oligonucleotide complex technique. In schemes 6, 8 and 10 are shown the hypothetical site-specific insertion/deletion mechanism at the targeted regions of RNA (or DNA) with the two step oligonucleotide complex technique. RNA (such as Huntington's disease) in Scheme 6 contains reiteration of excess triplets, and accordingly the purpose of the deletion step is to cut out the excess repeats. RNA shown in Scheme 8 is a model of active (harmful) RNAs and the goal of the insertion is to switch off these RNAs. The purpose of the insertion shown in Scheme 10 (opposite of Scheme 8) is to "wake up" a silent gene or to create new one. The insertion shown in Schemes 2, 4, 6, 8 and 10 could proceed with similar mechanisms, but goals achieved for each of the approach would be different.

The purpose of the approaches shown in Schemes 5, 7 and 9 is identical to the Schemes 4, 6 and 8. The deletion/insertion mechanism of the targeted region of RNA, shown on Schemes 5, 7 and 9, proceeds with the one step oligonucleotide complex technique, as described for Scheme 3.

In Schemes 11 and 12 are shown schemes for repair of an inactive tyrosinase gene by one and two step oligonucleotide complex approaches, respectively. The schemes contain one of the possible versions of the oligonucleotide complex which may be used for the replacement of mutated C with G in inactive tyrosinase mRNA. This type of alteration could achieve a phenotypic restoration of the mutated tyrosinase gene.

In Schemes 13 and 14 are shown schemes for repair of Cystic Fibrosis Δ508 mRNA by one and two step oligonucleotide complex treatment approach. As was described herein, mutated Δ508 mRNA contains two defects: first, absence of a UUU triplet at Δ508 region and change of U to a C immediately 5' to the same Δ508 position. Because of the close location of both mutations one set of oligonucleotide complex was sufficient for repair of both defects.

In some cases the targeted gene might contain more then one mismatch or inserted (deleted) region, which may be distantly located. For restoration of such genes it would be appropriate to use two or more oligonucleotide complex sets consecutively or simultaneously.

For enhancing targeted gene repair efficiency of oligonucleotide complex, they could be used synergistically with other known or potential therapeutic compounds.

The Examples herein show use of oligonucleotide complex compounds and methods for the restoration of a cystic fibrosis phenotype in a cultured Δ508CFTR cell line using hybrid oligonucleotide and a modified chimera of an RNA/RNA (DNA) duplex.

Loss or suppression of deleterious gene function by antisense oligonucleotide technologies involves specific inhibition of DNA and RNA synthesis and protein expression [Zamecnik et al., (1978), Proc. Natl. Acad. Sci. USA 74, 280-284; Stephenson et al., (1978) Proc. Natl. Acad. Sci. USA 75, 285-288]. Upon cell entry, exogenous complementary deoxyoligonucleotides hybridize with a target mRNA, inducing an excision endonucleolytic effect on the mRNA, due to activation of ribonuclease H (RNAse H) [Agrawal et al., (1990). Proc. Natl. Acad. Sci. USA 87, 1401-1405.], resulting in synergism with hybridization inhibition [Zamecnik et al., (1978), Proc. Natl. Acad. Sci. USA 74, 280-284; Zamecnik et al., (1996) Ed. By Sudhir Agrawal, Humana Press, NJ, 1-11]. Triplex-forming oligonucleotides have also been used to modify gene function [Felsenfeld et al., (1957) J. Am. Chem. Soc., 79, 2023-2024; Thoung et al., (1993) Angewandte Chemie. Intl. Ed. Eng., 32, 666-690]. Likewise, RNA editing [Simpson et al., (1996) Annu. Rev. Neurosci. 19, 27-52] has been reported in numerous cell systems where nucleotide sequences can also be modified at the RNA level. Other mechanisms may apply to dsRNA sequences if introduced into biological systems. This is the focus of current attention for their role in RNA interference (RNAi) and gene silencing at the transcriptional level [Hannon, (2002) Nature, 418, 244-251]. RNAi mediated gene silencing was discovered in *C. elegans* [Fire et al., (1998) Nature, 391, 806-811], and also found in plants and mammals [Zamore et al., (2000) Cell, 101, 25-33]. Gene silencing results from the successive cleavage of

long dsRNA to oligonucleotide small interfering RNAs (siRNAs) by DICER enzymes [Zamore et al., (2000) Cell, 101, 25-33]. After oligonucleotide hybridization of siRNA with mRNA, a common feature shared with antisense DNA or RNA, the cleavage of target mRNA is catalyzed by an enzyme different from RNase H [Zamore et al., (2000) Cell, 101, 25-33]. Separate from the above is CpG stimulation of the host immuno-modulatory mechanisms, which has inhibitory implications in both prokaryotic and eukaryotic organisms [Krieg (2003) Nat. Med., 9, 831-835; Kandimalla et al., (2003) Biochemical Society Transactions, 31, 654-658].

Gain of function and/or correction of defective genes present a further challenge for gene therapy. Partial restoration of Δ508 CFTR function may be induced by membrane insertion of the mutated channel without changes in either phenotype or genotype [Brown et al., (1996) Cell Stress & Chaperones, 1, 117-125; Arispe et al., (1998) J. Biol. Chem. 273, 5727-3574]. RNA/DNA oligonucleotide hybrids [Yoon at al., (1996) Proc. Natl. Acad. Sci. USA 93, 2071-2076 ; Parekh-Olmedo et al., (2001) Sci. STKE 73, PL1] and single-stranded oligonucleotides [Igoucheva et al., (2001) Gene Therapy, 8, 391-399] have also been used for correction of defective genes. Substituted, circular or single stranded RNA/DNA chimeras have been employed to insert base pairs in deficient genomic DNA [Yoon at al., (1996) Proc. Natl. Acad. Sci. USA 93, 2071-2076 ; Parekh-Olmedo et al., (2001) Sci. STKE 73, PL1]. Some attempts at replicating this work, however, showed no nucleotide exchange in the targeted loci by cloning of the PCR products [Zhang et al., (1998) Antisense & Nucleic Acid Drug Developm. 8, 531-536], and these results were viewed as PCR artifacts created by the RNA/DNA oligonucleotides themselves [Zhang et al., (1998) Antisense & Nucleic Acid Drug Developm. 8, 531-536; Taubes, (2002) Science, 298, 2116-2120]. Transcriptional repair is also the focus of recent attention in the repair of mRNA. Spliceosome mediated RNA transplicing (SmaRT) technology has been used to modify Δ508 CFTR transcripts in human CF airway epithelia [Liu et al., (2002) Nat. Biotechnol. 20, 47-52].

We previously observed, as well as others, that mRNA, hybridized to a single stranded synthetic short piece of DNA, activates RNase H, a ubiquitous enzyme that hydrolyzes specifically the hybridized segment of mRNA [Agrawal et al., (1990). Proc. Natl. Acad. Sci. USA 87, 1401-1405]. At least a 4-5 nucleotide internally located region, hybridized to a phosphorothioate modified (PS) oligodeoxyribonucleotide, is needed for the excision [Agrawal et al., (1990). Proc. Natl. Acad. Sci. USA 87, 1401-1405].

In this report, single complementary strand oligonucleotides were constructed and annealed to form a duplex. Duplexes were added to growing Δ508 CFTR cell cultures, and patch clamping studies [Reisin et al., (1994) J. Biol. Chem. 269, 20584-20591] showed evidence of phenotypic reversion of cells to a normal phenotype, namely cAMP-activated anion currents. Phenotypic recovery was confirmed by protein kinase A (PKA)-activated single channel currents and SPQ Cl^- fluorescence. Nucleotide treated cells contained an insertion of missing bases in the CFTR mRNA. The mechanism of insertion into mRNA is still under investigation.

EXAMPLES

The following Material and Methods were used throughout the Examples.

Synthesis of oligonucleotides.

DNA and RNA oligonucleotides (Table 1) were synthesized on a 394 DNA/RNA synthesizer (Applied Biosystems) with phosphoramidite chemistry and standard phosphoramidite monomers from Glen Research. For the introduction of 3' and 5' phosphate groups on the CF4 oligonucleotide (Table 1), 5'- and 3'-phosphorylation reagents were used accordingly (Glen Research). Phosphorothioate bonds were introduced by sulfurization with Beaucage thiolating reagent [Padmapriya et al., (1994) Antisense Res. Dev. 4, 185-199]. 2'-0-methyl modifications, oligoribonucleotides and phosphorothioate oligonucleotides were synthesized, and HPLC purified as described [Metelev et al., (1994) Bioorg. Med. Chem. Lett. 4, 2929-2934; Agrawal et al., (1998) Antisense Nucleic Acid Drug Dev. 8, 135-139].

Annealing of CF4/CF6 oligonucleotide duplex.

Stock solutions of CF4 (1mM) and CF6 (1mM) were prepared by dissolving compounds in high ionic strength buffer (0.2 M NaCl, 20 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.0). Duplex formation was prepared with 1:1 (v:v) mixture of compounds, by heating to 75-80°C, and cooling down gradually to room temperature. All duplexes and compounds were sterilized by passage through 0.45 μm cellulose acetate centrifuge filters (Costar).

Cell culture and incubation procedures.

Mouse mammary carcinoma cells (c127i) transfected with human epithelial CFTR wild type (WT1) or Δ508 CFTR cells [Cantiello et al., (1994), J. Biol. Chem. 269, 11224-11232; Dechecchi et al., (1993) J. Biol. Chem. 268, 11321-11325] were used for these studies. The transfected c127i cells are high level producers of Δ508 CFTR mRNA. Cells were grown and

maintained in Dulbecco's medium (DMEM), supplemented with 10% fetal bovine serum and 1% L-glutamine, as previously reported [Verkman, (1990) Am. J. Physiol. 259, C375-C388].

Electrophysiology and chemicals.

Whole-cell and excised inside-out patches were obtained to assess cAMP-PKA dependent anion currents in treated Δ508 cells. Currents and command voltages were obtained and driven. respectively, with a Dagan 3900 amplifier using a 1 gigaohm headstage. The excised patch-clamp configuration was carried out as previously described [Reisin et al., (1994) J. Biol. Chem. 269, 20584-20591]. Single channel data were obtained between ±100 mV in symmetrical Cl^-.Data were further analyzed as previously described [Reisin et al., (1994) J. Biol. Chem. 269, 20584-20591]. The pipette and bathing solution contained, in mmol/L, either: NaCl 140, $MgCl_2$ 1.0, KCl 5, and N-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES) 10, at pH 7.4, or $MgCl_2$ 70, HEPES 10, pH 7.4. The bathing solution also contained 1.0 mmol/L $CaCl_2$. Whenever indicated, the patch-pipette was filled up to at least one third of its height with either MgATP or TrisATP (100 mmol/L, pH 7.4 adjusted with N-methyl-glucamine) as previously reported [Reisin et al., (1994) J. Biol. Chem. 269, 20584-20591]. Experiments were conducted at room temperature. The cAMP stimulatory mixture contained 8-Br-cAMP, isobutyl-methyl-xanthine (IBMX) and forskolin. Final concentrations were 500 μmol/L, 200 mol/L, and 10 μmol/L, respectively. The catalytic subunit of PKA was used at a final concentration of 20 μg/ml. The Cl^- channel blocker, diphenylamine-2-carboxylate (DPC) was kept in a 100-fold stock solution (20 mmol/L) in 50 % water/ethanol. DPC was used at a final concentration of 500 μM.

SPQ fluorescence technique.

Cyclic-AMP-stimulated Cl^- transport was also followed by fluorescence changes of cells loaded with the Cl^- sensitive dye 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) [Verkman, (1990) Am. J. Physiol. 259, C375-C388]. Briefly, cells were grown to partial confluence on glass coverslips. SPQ cell loading was conducted by a15-min incubation in a Cl^- free diluted salt solution containing SPQ (5 mM). The saline solution contained 135 mM Na^+-gluconate, 2.0 mM KH_2PO_4, 1.0 mM $MgSO_4$, 1.0 mM Ca^{2+}-gluconate, and 10 mM HEPES (pH 7.4). In some experiments, gluconate was replaced by either isethionate or aspartate with similar results. SPQ fluorescence was determined under an oil immersion X20 objective in a Nikon E-800 fluorescence microscope (Tokyo, Japan), with an UV filter (96101C UV-2E/C). Images were captured with a Hamamatsu (C4742-95) digital camera and stored as TIFF files in a Macintosh

computer with the IPLab Spectrum software (Signal Analysis Corp.). Pictures were analyzed digitally with the NIH Image 1.62b7 software. SPQ cytoplasmic fluorescence values were normalized between intra-nuclear (considered as zero quenching, i.e. low Cl^-), and extracellular background. Data were expressed as percent fluorescence with respect to time zero, the time when cells were placed in an isotonic saline solution. The solution contained 135 mM NaCl, 2.0 mM KH_2PO_4, 1.0 mM $MgSO_4$, 1.0 mM Ca^{2+}-gluconate, 10 mM HEPES (pH 7.4), with or without the cAMP stimulatory combination mixture.

Primers and RT-PCR of Δ508CFTR mRNA.

Total RNA isolated by Trizol® reagent according to the manufacturer's protocol (Invitrogen, CA) from either WT1 or Δ508 cells treated with or without CF6/CF4 annealed duplex oligonucleotides (final concentration of duplex in reaction mixture 10 μM) was used to perform the RT-PCR assay [Kleppe et al., (1971) J. Mol. Biol. 56, 341-361] in two steps by using a ThermoScript RT-PCR system (GIBCO, BRL). Several sets of primers were used (see CF Table 1). In the first step, total RNA (~ 2 μg) was incubated for 60 min at 55°C with amplification refractory mutation system (ARMS) [Ferrie et al., (1992) Am. J. Hum. Genet. 51, 251-262] reverse mutant (M1) and normal (N1) primers (0.5 μg each) separately for the first-strand synthesis. In a second step, after heating at 94°C for 5 min, 35 cycles of PCR were carried out on the samples. PCR cycles were performed first by denaturation at 94°C for 2 min, annealing at 62°C for 1 min, and extension at 72°C for 2 min, followed by a final extension for 10 min at 72°C. Two ARMS reverse primers (N1 and M1) [Ferrie et al., (1992) Am. J. Hum. Genet. 51, 251-262], and a forward (F1) primer were used (Table 1). Similarly RT-PCR was performed with ARMS PS reverse primer (SCFR) and PS forward primer (SNF1, Table1). RT-PCR products were separated in 3% agarose gel and subjected to automated DNA sequence analysis.

Similarly, RT-PCR was conducted using allele specific primers (Scheme 1B, Table 1) as follows. In the first step, total RNA (~2 μg) was incubated for 60 min at 55°C with reverse (CFR) primer (0.5 μg each) for the first-strand synthesis. In a second step, samples were heated at 94°C for 1 min. PCR was performed for thirty cycles at 60°C including denaturation at 94°C (45 sec), annealing at 60°C (45 sec), and extension at 72°C for 1 min. This procedure was ended with a seven-minute final extension at 72°C. Two forward primers (CFW and CFM) and one reverse primer (CFR) were used for allele specific RT-PCR (Table 1). The RT-PCR assay was

performed with the MasterAmp™RT-PCR system (Epicentre, WI). In this procedure, combined reverse transcription and PCR were performed in the presence of forward normal (NF1) and mutant (MF2) and reverse (CFR) primers (0.5 μg each) separately. First, samples were incubated for 20 min at 60°C. PCR was performed for 40 cycles at 94°C, denaturation for 30 sec, annealing at 62°C for 30 sec and 72°C extension for 1 min. The reaction ended by a final extension for 6 min at 72°C. Two forward primers (NF1 and MF2) and a reverse primer (CFR) were used for this procedure. RT-PCR products were separated in a 3% agarose gel and were then subjected to automated DNA sequence analysis. ThermoScript RT-PCR products obtained by ARMS primers were subcloned for further separation and purification of possible heterogeneity of oligomers so obtained in pCR-Blunt vector, according to the manufacturer's protocol (Invitrogen, CA). The resulting clones were subjected to automated DNA sequence analysis using a T7 promoter primer.

Example 1. Restoration of Δ508CFTR phenotype.

To restore the normal (wild type) phenotype in cells expressing Δ508CFTR, modified oligonucleotides were constructed and their effect on ion transport was assessed in Δ508 cells treated with these constructs. In a first step, a modified oligodeoxyribonucleotide was synthesized, containing phosphorothioate (PS) and PS plus 2'-O-methyl modifications for the central and flanking segments, respectively. This oligonucleotide was designed to hybridize in Watson-Crick base complementarity to the region of Δ508 mRNA where the PS section would be directly opposite the Δ508 mRNA position, flanked 3'- and 5'- by adjacent nucleotides complementary to the Δ508 mRNA. Accordingly, the PS segment would activate endogenous RNase H, and cleave those bases opposite the PS complementary oligonucleotide (10 μM). The insertion of bases then occurs in a second step.

For the second or insertion step, two complementary single strand oligonucleotides, CF4 and CF6 (Table 1, Scheme 1A) were constructed. The annealed CF4/CF6 duplex (10 μM final concentration) was added to the Δ508 CFTR cells after 2 hours incubation, then washout, in the deletion step. Next, following overnight or up to 72 hour incubation in the second (insertion) step, patch clamp examination was conducted on the treated cells.

Whole cell currents of two step treated Δ508 cells (Fig. 1) showed a 243% increase after cAMP stimulation (2.98 ± 0.68 nS/Cell vs. 0.87 ± 0.16 nS/Cell, n=24, $p<0.01$), which was absent in the control Δ508 cells (Fig. 1). The cAMP-activated currents were largely (>84%) inhibited by the CFTR inhibitor DPC (500 μM) as expected for wild type CFTR. Similar results were obtained with WT1 cells, over-expressing wild type CFTR in the same cellular background. The treated Δ508 cells (10/12) displayed PKA and ATP activated 10-12 pS Cl^- channels (Fig. 2) not observed in the control Δ508 cells (0/24).

When the CF4/CF6 annealed duplex was, however, added to the tissue culture cells without performing a first oligonucleotide treatment step as described above, phenotypic reversion was likewise found, as determined by the patch clamp technique. We therefore chose to pursue one step phenotypic reversion technique as a simpler model for the sequencing studies described below.

Surprisingly, CF4/CF6 cells sporadically showed large, DPC inhibitable whole-cell currents in the absence of cAMP simulation (data not shown). Both WT1 and CF4/CF6 treated Δ508 cells responded with a comparably similar change in SPQ fluorescence in response to cAMP stimulation (Fig. 3) [Ram et al., (1989) Proc. Natl. Acad. Sci. USA 86, 10166-10170]. Control Δ508 cells showed no cAMP-induced change in SPQ fluorescence (Fig. 3). The data indicate that CF4/CF6 treatment of Δ508 cells restores a normal tissue culture phenotype, consistent with the presence of functional CFTR.

Example 2. Sequencing analysis of PCR products.

In order to determine the extent of restored phenotype in the CF4/CF6 treated Δ508 cells, total RNA was isolated and CFTR specific primers were used to amplify the predicted region. CFTR wild type specific primers efficiently amplified single bands of the expected size in the CF4/CF6 oligonucleotide treated cells. The sequence region after this treatment did not show changes in the PCR amplified oligonucleotides when wild type primers were used. Because the number of Δ508 mRNA copies that are potentially repaired may be low, allele specific primers for either wild type or mutated CFTR were used next (CFFW and CFFM, Table 1).

Allele specific primers detected mRNA from serial dilutions of wild and Δ508 total RNA by means of a shift of the amplified band (Fig. 4). Wild type RT-PCR product could be detected in 1:10,000 dilutions in Δ508 mRNA background (Fig. 4). Initially, RT-PCR product from the

allele specific wild type primer (CFFW, Table 1) in the oligonucleotide treated Δ508 cells also failed to detect insertion in the amplified band. Therefore, total RNA of CF4/CF6 treated Δ508 cells was further tested by PCR analysis with ARMS specific primers (Fig. 5) [Skerra, (1992) Nucleic Acid Res. 20, 3551-3554]. Wild type and mutated ARMS primer-amplified PCR bands were examined by DNA sequence analysis. The sequence of the amplified mutated cDNA showed a variety of one-codon insertions, rich in G residues. Whether a GGG codon (glycine) is an acceptable substitute for phenylalanine remains to be determined, since systematic study of such base insertion has not been done to our knowledge.

To analyze this observation further, the PCR products were subcloned in pCR-Blunt vector and subjected to DNA sequence analysis. The fraction of control RNAs isolated from untreated Δ508 cells showed no oligonucleotide insertion. However, mRNA isolated from CF4/CF6 treated Δ508 cells showed 20-30% UGU insertion, based on analysis of the percentage of subclones showing TGT insertion into the RT-PCR generated Δ508 DNA. This percentage of insertion is apparently sufficient for phenotypic reversion in the tissue culture system. None of the subcloned untreated Δ508 cells displayed false positives (three-base insertion) in the region flanking the initial deletion (Fig. 6). Combined reverse transcription and PCR were performed with forward wild type (NF1) and mutant (MF2), and reverse (CFR) primers (0.5 μg each) separately. Insertion of bases in the proper position (Scheme 1B) has been found in subcloned and sequenced RT-PCR products, from treated but not control Δ508 cells.

Without being limited by any particular mechanism, the hypothesis was tested as to whether base insertion in the treated Δ508 cells has occurred at the RNA level. It was possible that the repair mechanism was carried out instead of/or in addition to, the DNA level, by way of reverse transcription extending back to the genome. This was tested by RT-PCR analysis of DNA from subcloned Δ508 cells originally treated with CF4/CF6. No evidence was obtained that the restored phenotype was carried back to the inheritable DNA genome level was obtained. Western blot analysis was conducted with antibodies targeted to epitopes upstream and downstream of the Δ508 deletion, respectively. The data indicate that the full-length protein was made in the presence of the oligonucleotides (data not shown).

Further, the data rule out that partially degraded protein was being translated after treatment with the oligonucleotides because of stop codon missing signals in the treated mRNA

[Bedwell et al., (1997) Nature Med., 3, 1280-1284]. Both antibodies showed the same level of protein, without shorter, truncated or degraded peptides. Thus, treatment with CF4/CF6 does not act as an inhibitor of protein synthesis.

Example 3. Mechanism of insertion.

As an initial step, restoration was sought of the most common CF phenotype by antisense oligodeoxynucleotide hybridization to the region immediately adjacent to the trinucleotide deletion, on both sides of the Δ508CFTR mRNA. The double-stranded synthetic 2'-O-methyl-RNA/unmodified-RNA oligonucleotide chimera (CF4/CF6) was constructed and used to anneal, selectively cut, and repair the missing region (Scheme 1B). This duplex, as shown, contains a single stranded 2'-O-methyl-substituted 33-mer oligoribonucleotide (CF4) hybridized to an unmodified 11-mer oligoribonucleotide with 5' and 3' monophosphate termini (CF6). The role of the 5'- and 3'- phosphate termini of CF6, if any, remains to be determined. The CF4/CF6 chimera may theoretically hybridize with the mRNA bearing the deletion (Scheme 1B), followed by an mRNA cleavage step, as a result of which CF6 might theoretically be spliced into this region or alternatively, serve as a triplex backbone sequence for a one-by-one insertion mechanism.

Although insertion of missing bases in the deficient mRNA was found, as shown supra, the specific molecular mechanism is uncertain. Without being limited by any particular mechanism, the mismatch created by using the oligonucleotides provided herein and found immediately proximal in the 5'-direction to the Δ508 mRNA UUU deletion may induce an as yet unknown deletion mechanism. For one, the deletion step may be induced by either a new enzyme or by the Dicer enzyme. Another possibility is that RNAse H, in the presence of a triplex, may induce the deletion. There may conceivably be a deletion in RNA analogous to the MutS DNA mismatch mechanism [Wang et al., (2003) Proc. Natl. Acad. Sci. USA 100, 14822-14827]. The process by which CF4/CF6 restores the phenotype is consistent with specific docking and cleavage of selective nucleotide sequences hybridized to an mRNA sequence. A -TTT- insertion into the PCR amplified deoxyoligonucleotide would be expected, if the -UUU- from CF6 had been inserted into the Δ508 mRNA. However, TGT (UGU in the Δ508 mRNA) insertion was consistently observed in clones obtained by ARMS primers amplification RT-PCR products (Scheme 1B and Fig. 6). This finding suggests a one-by-one insertion mechanism, with a G in place of a U.

Initial sequencing of RT-PCR oligodeoxynucleotides obtained with ARMS primers specific for the CFTR wild type sequence (N1, Table 1) revealed the presence of a mixture of residues rich in G's in the Δ508 site. Subcloning of this oligodeoxynucleotide band resulted in the finding that some but not all of the sequenced cDNA material expressed a TGT at this Δ508 site. Since it was mRNA that was subjected to RT-PCR amplification, the corresponding bases in the Δ508 region were actually UGU. The G residue in the UGU could be accounted for by a restoration mechanism with other than Watson-Crick complementarity in insertion [Yang et al., (2003) Proc. Natl. Acad. Sci. USA 100, 15376-15380]. The 3' hybridization initial steps enabled by the ARMS primers may also be flawed by exonucleotide-induced primer degradation in the reverse transcriptase step of the RT-PCR amplification procedure [Skerra, (1992) Nucleic Acid Res. 20, 3551-3554; Smith at al., (2003) Proc. Natl. Acad. Sci. USA 100, 15440-15445]. Other possibilities of error introduction [Kobayashi et al., (1990) Am. J. Hum. Genet. 47, 611-615], may explain the apparently artifactual deletions shown in Scheme 1B which accompany insertions. This consideration was partially confirmed by wild type ARMS primer-RT-PCR product amplification of the sequence in WT1 cells, which only carries wild type CFTR. In this reaction, the expected -TTT-was found by sequence analysis of the wild type RT-PCR material, in contrast to the TGT (actually UGU in mRNA) when the repaired Δ508 mRNA was sequenced.

For further clarification of the above results, new sets of primers were constructed with an initial phosphorothioate substituted nucleotide in the 3' end, followed by several (PO) standard nucleotides. This improved primer selectively amplified CF4/CF6 treated, but not control (untreated) RT-PCR product in Δ508 cells. The ARMS forward PS primer (SNF1) inserts UGU without a concomitant new deletion (Scheme 1B, Table 1), thus presenting the best case for phenotypic reversion to wild type. Just 5' to the UUU in the wild type CFTR is a C residue, while in the mutant Δ508 CFTR this residue is a U. This may conceivably result in replacement failure 5' to the inserted UGU using the ARMS reverse primer (N1). The purpose in using CF4/CF6 complex, which has a G residue rather than U proximal to the 5' end of the Δ508 deletion, was to make this residue complementary to that in the wild type sequence rather than in the deleted Δ508 sequence. Both AUC and AUU code for isoleucine. This base mismatch for the Δ508 mRNA (CF Scheme 1) may however induce a single strand break, analogous to that found for single DNA mismatches as mentioned above [Wang et al., (2003) Proc. Natl. Acad. Sci. USA 100, 14822-14827], necessary for a subsequent repair mechanism to be initiated. This eliminates

the possibility that the change of C to a U, immediately 5' to the TTT in the same position in the Δ508 gene may contribute, in addition to the Δ508 TTT deletion, to the phenotypic change in CFTR.

DNA polymerase has a high degree of Watson-Crick fidelity in synthesizing complementary strands. Reverse transcriptase, which starts at the 3'-end of the PCR amplification, however, has a lower level of this specific type of fidelity. This property of reverse transcriptase may be a possible explanation for the UGU (the equivalent of TGT) found in the amplified, restored Δ508 mRNA. In relation to phenotypic restoration, benign mutations of the TTT present in the wild type gene do exist. TGT is one of these, coding for cysteine, which appears to be an acceptable substitute for phenylalanine in the Δ508 region [Kobayashi et al., (1990) Am. J. Hum. Genet. 47, 611-615]. The 2'-O-methyl group, plus the 5-methyl of thymidine, which uridine does not have, may also alter the tertiary structure of the CF4 chimera. Such factors may influence the nucleophilicity, electrophilicity, and polarizability of the bases, which make up the mRNA-CF4/CF6 triple-stranded structure. Tinoco and colleagues have described numerous double stranded DNA/RNA base complementarities which conformational and other experimental molecular conditions may cause to favor over the standard Watson-Crick AT and GC ones [Burkard at al., (1999) *In* The RNA World. editors. Cold Spring Harbor Lab. Press, New York. 675-680]. The highly sensitive nanosphere/gold procedure of Letsinger and colleagues [Taton et al., (2000) Science, 289, 1757-1759; Letsinger at al., (2000) Bioconj. Chem. 1, 289-291] may in the future be applicable as an alternative to the PCR technique, avoiding possible artifacts introduced by PCR amplification, or else may be used following subcloning.

The data herein indicate that specific base insertion in Δ508 mRNA has been made. Certain PCR-introduced artifacts have been avoided by phosphorothioate modification of the 3'-terminal residues of ARMS specific primers. Under our best conditions thus far, insertion of UGU has taken place in a sufficient fraction of Δ508 mRNA to induce phenotypic but not genotypic reversion in a tissue culture cell line. The subcloning data shown 20-30 percent insertion of UGU in the site of the Δ508 mRNA UUU triplet deletion.

What is claimed is:

1. A method for targeted gene repair, comprising

contacting a genomic target DNA of a cell with a hybrid RNA oligonucleotide complex, said complex comprising a first strand and a second strand, said first strand comprising a flanking sequence complementary to said target DNA and central sequence comprising at least one non-complementary nucleotide in a location opposite a defect of said target DNA, wherein said flanking sequence of said first strand comprises an RNase H-resistant modification 3' and 5' to said non-complemenary nucleotide, wherein said second strand is shorter than said first strand, complementary to said first strand and annealed thereto; and

hybridizing said complex to said target DNA, wherein a repaired RNA is produced, said RNA comprising a sequence alteration opposite said defect of said target DNA, wherein said genomic target DNA sequence is unaltered.

2. The method of claim 1, wherein said alteration is not maintained in progeny of said cell.

3. The method of claim 1, wherein said defect is a substititution, deletion, or addition of a base pair compared to a normal wild type sequence.

4. The method of claim 1, wherein said RNase H-resistant modification is the addition of a 2-O-methyl moiety.

5. The method of claim 1, wherein at least one nucleotide of said flanking sequence 3' to said non-complementary base pair and at least one nucleotide of said flanking sequence 5' to said non-complementary base pair comprise said RNase H-resistant modification.

6. The method of claim 1, wherein said first strand comprises a phosphorothioate linkage.

7. The method of claim 1, wherein said first strand is at least 15 nucleotides in length.

8. The method of claim 1, wherein said first strand comprises at least 16 nucleotides.

9. The method of claim 1, wherein said flanking sequence of said first strand comprises at least two nucleotides.

10. The method of claim 1, wherein said second strand comprises at least 7 nucleotides.

Table 1. Sequences of oligonucleotides used (5'>3')

	CF4	uuu cuu uua uag uag aaa cca caa agg aua cua (SEQ ID NO: 1)
	CF6	pC AUC UUU GGU Gp (SEQ ID NO: 2)
	F1	**GGG AGA ACT GGA GCC TTC A** (SEQ ID NO: 3)
5	N1	**GTA TCT ATA TTC ATC ATA GGA AAC ACC ACA** (SEQ ID NO: 4)
	M1	**GTA TCT ATA TTC ATC ATA GGA AAC ACC ATT** (SEQ ID NO: 5)
	NF1	**GCC TGG CAC CAT TAA AGA AAA TAT CAT CTT** (SEQ ID NO: 6)
	MF2	**GCC TGG CAC CAT TAA AGA AAA TAT CAT TGG** (SEQ ID NO: 7)
	CFR	**GTT GGC ATG CTT TGA TGA CGC TTC** (SEQ ID NO: 8)
10	CFFW	**GGC ACC ATT AAA GAA AAT ATC ATC TT** (SEQ ID NO: 9)
	CFFM	**GGC ACC ATT AAA GAA AAT ATC ATT GG** (SEQ ID NO: 10)
	SCFR	**GTT GGC ATG CTT TGA TGA CGC TTC** (SEQ ID NO: 11)
	SNF1	**GCC TGG CAC CAT TAA AGA AAA TAT CAT CTT** (SEQ ID NO: 12)
	SMF2	**GCC TGG CAC CAT TAA AGA AAA TAT CAT TGG** (SEQ ID NO: 13)

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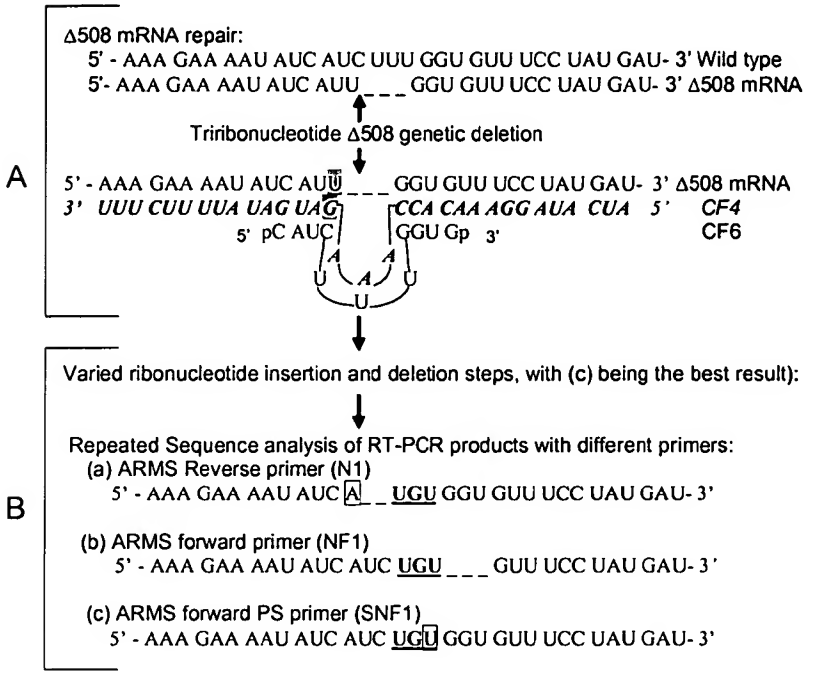
Table 1. Primers and constructs used to repair CFTR mRNA. Lower case letter are a mucleotide sequence in CF4 corresponding to bases of 2'-O-methylribosyl oligonucleotides, with internucleoside phosphate bonds. Upper case letter are a nucleotide sequence in CF6 corresponds to natural RNA sequences and small case p corresponds to the terminal phosphate groups. Bold
20 type corresponds to the natural DNA sequences. Shaded region in SCFR, SNF1 and SMF2 corresponds to internucleoside phosphorothioate bonds.

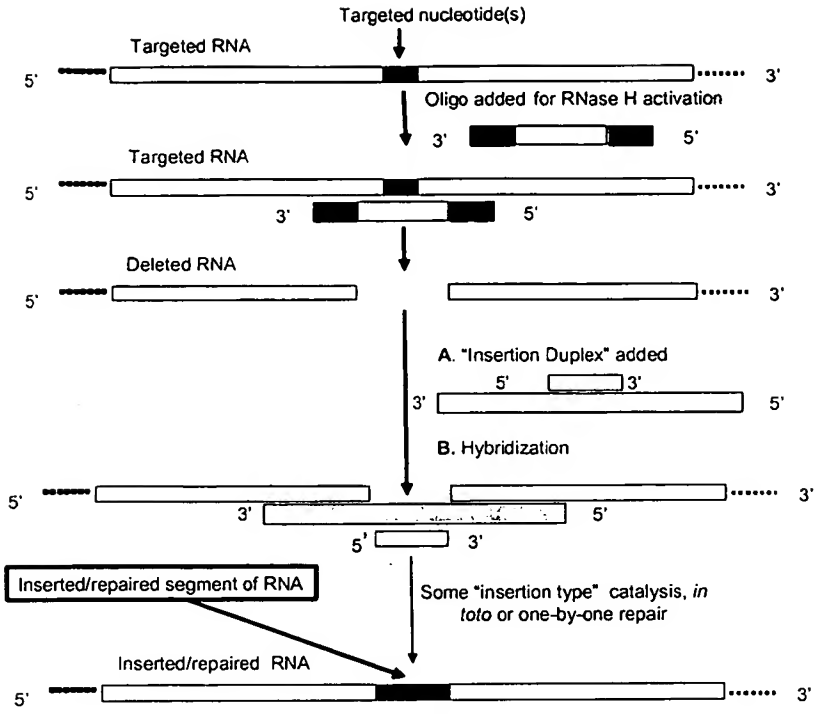
Abstract

Compositions and methods of treatments of cells are provided for altering the phenotype of a cell by administering an oligonucleotide complex to the cell, the complex having two strands and chemical modifications.

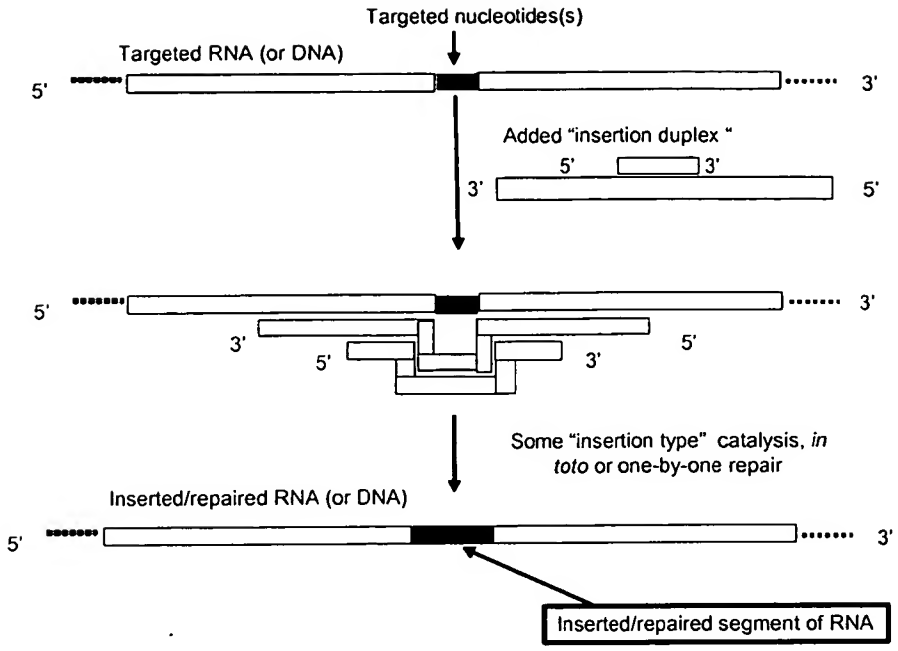
Scheme depicting deletion in Δ508 mRNA, possible configuration of CF4/CF6 in the hybridization step, and varied replacements which were found.



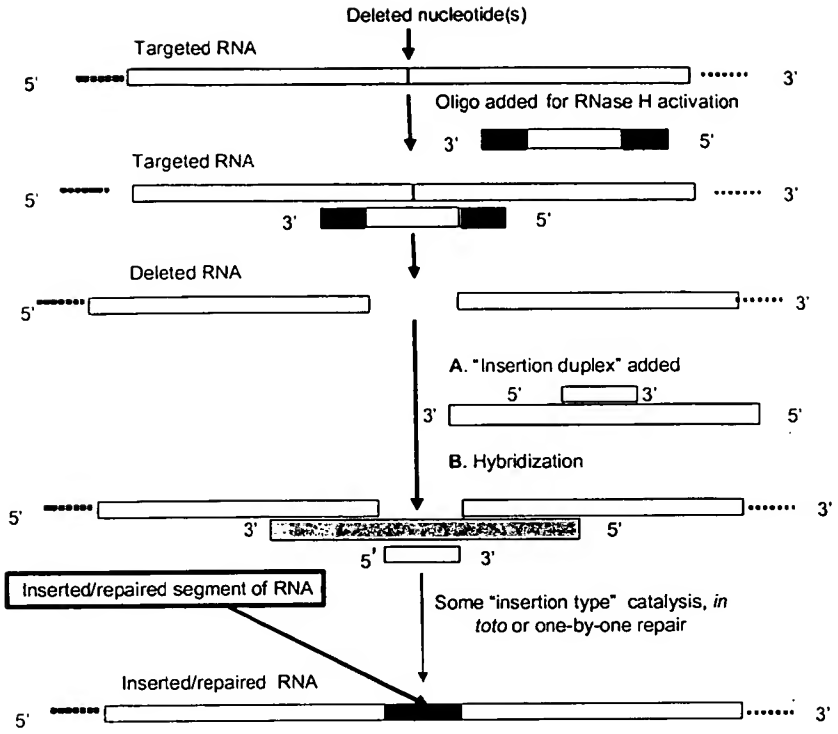
Scheme 1


Targeted nucleotide(s)
Targeted RNA
5'
3'
Oligo added for RNase H activation
3'
5'
Targeted RNA
5'
3'
3'
5'
Deleted RNA
5'
3'
A. "Insertion Duplex" added
5'
3'
3'
5'
B. Hybridization
5'
3'
3'
5'
5'
3'
Inserted/repaired segment of RNA
Some "insertion type" catalysis, *in toto* or one-by-one repair
Inserted/repaired RNA
5'
3'

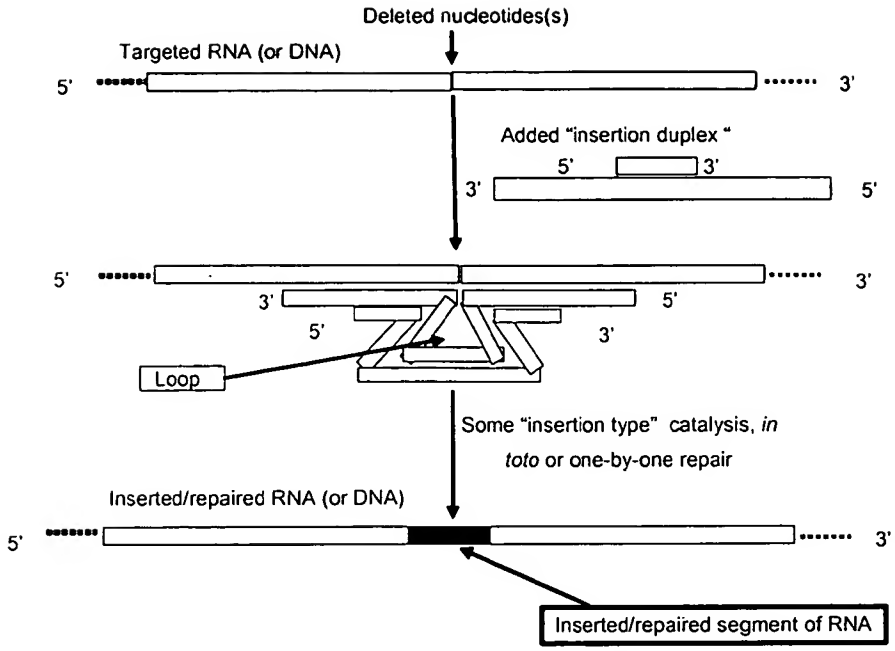
Scheme 2


Targeted nucleotides(s)
Targeted RNA (or DNA)
5'
3'
Added "insertion duplex "
5'
3'
3'
5'
5'
3'
3'
5'
5'
3'
Some "insertion type" catalysis, *in toto* or one-by-one repair
Inserted/repaired RNA (or DNA)
5'
3'
Inserted/repaired segment of RNA

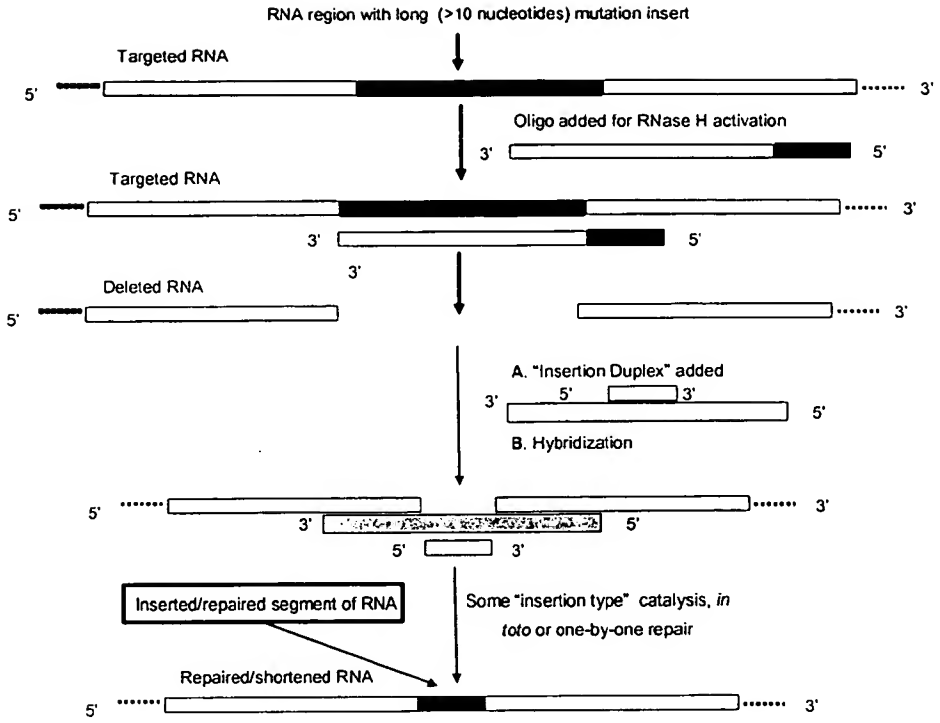
Scheme 3



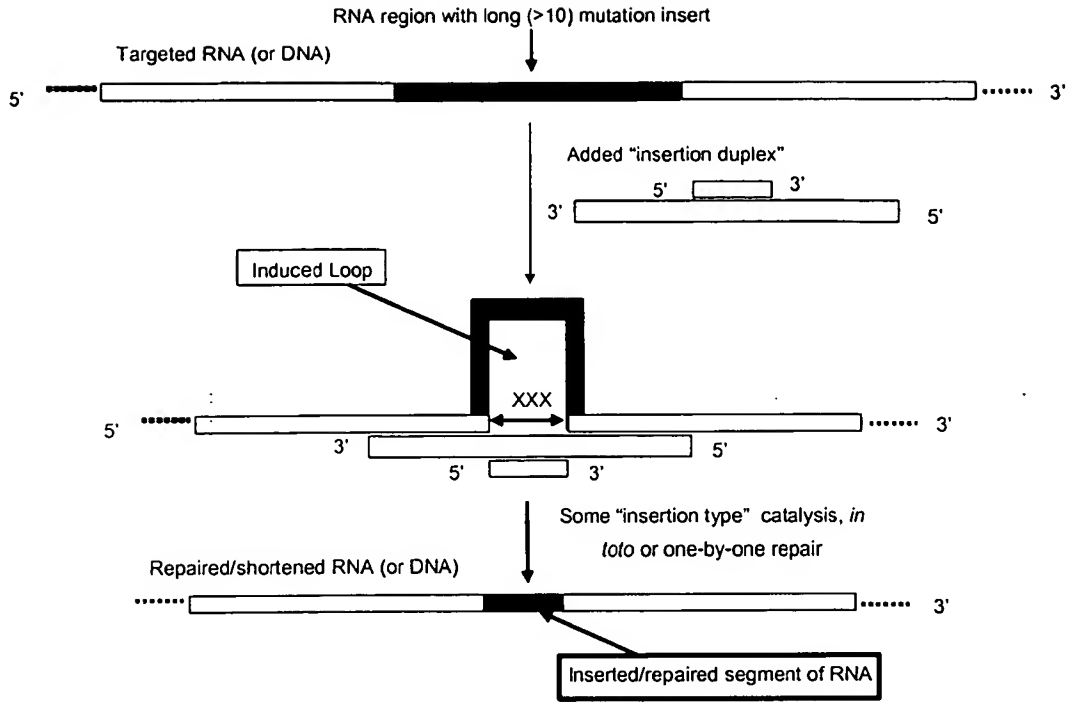
Scheme 4



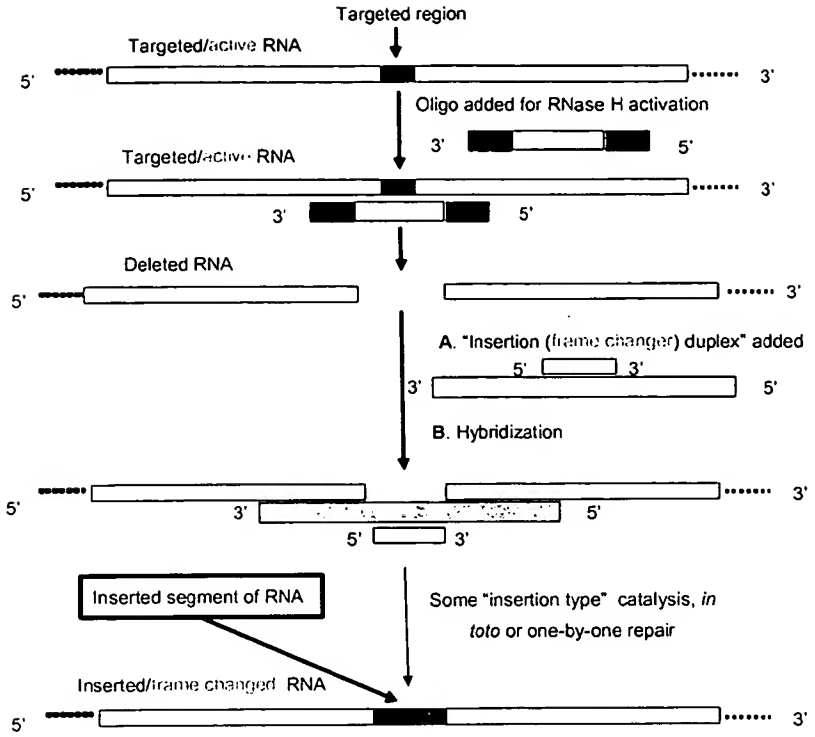
Scheme 5



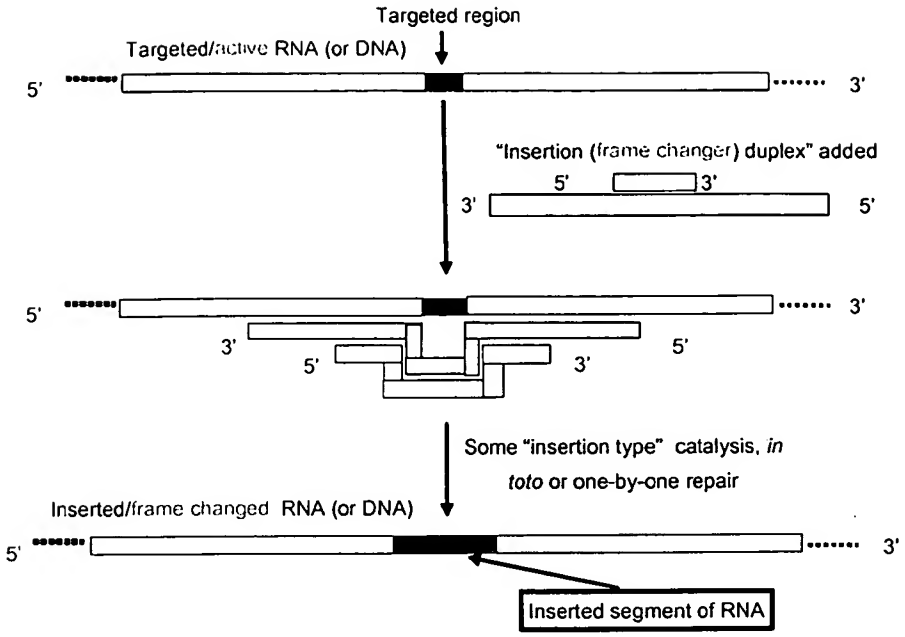
Scheme 6



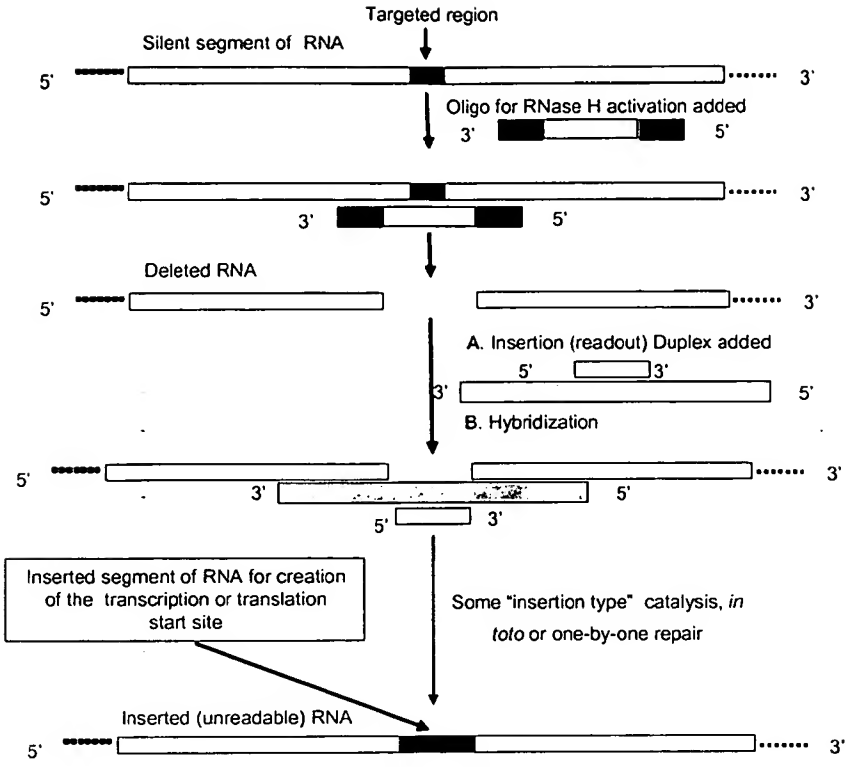
Scheme 7



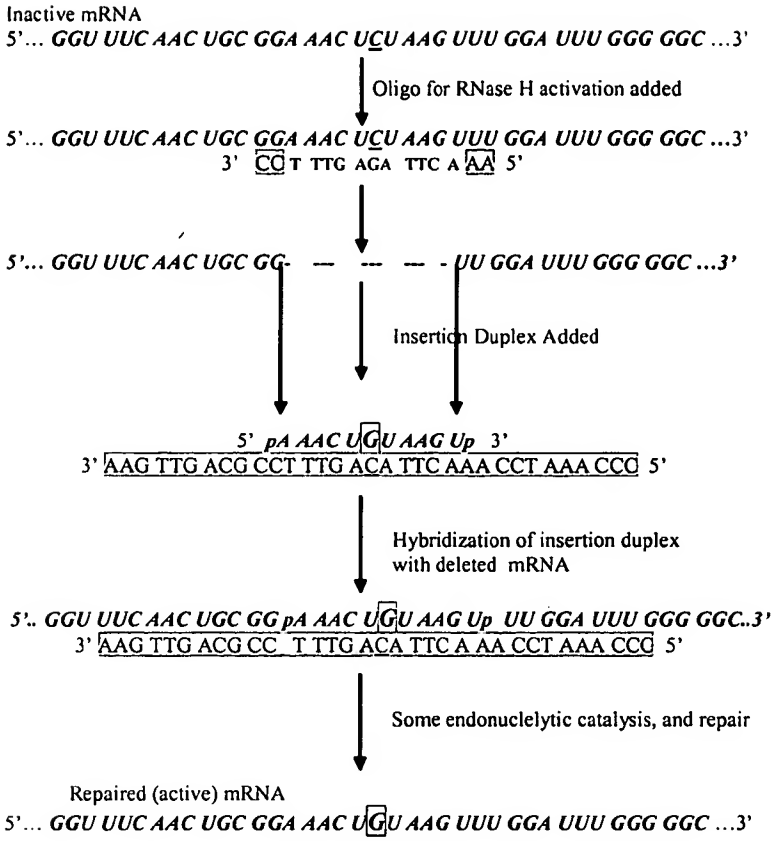
Scheme 8



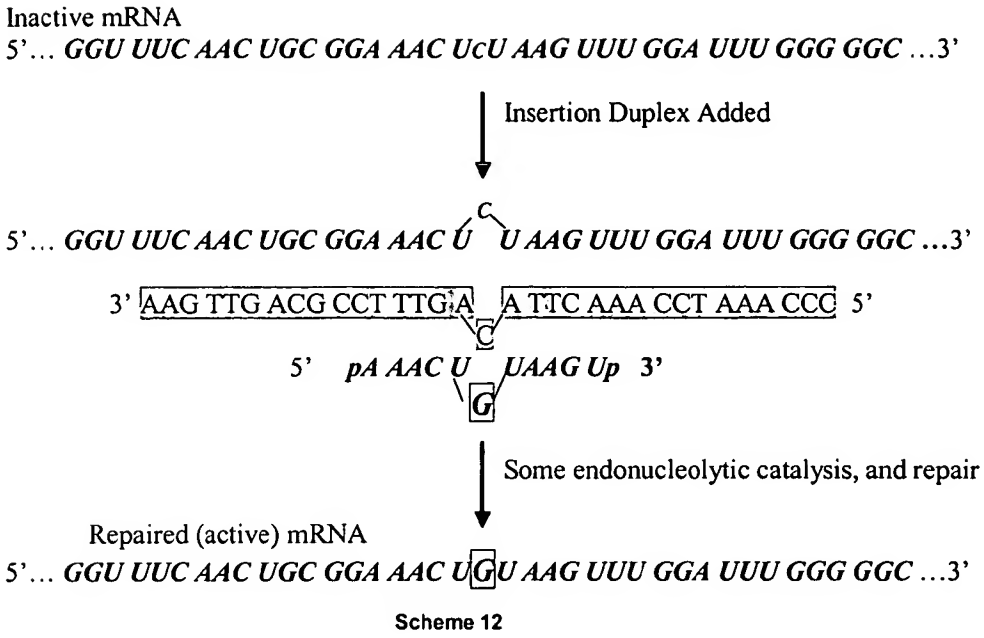
Scheme 9



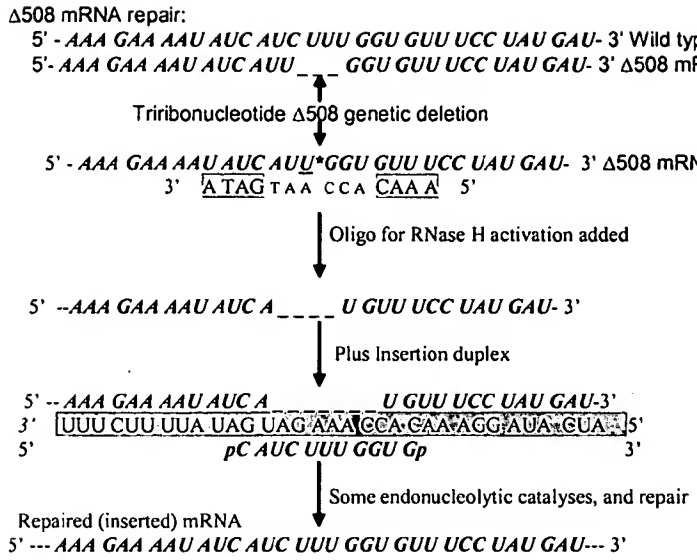
Scheme 10


Inactive mRNA
5'... GGU UUC AAC UGC GGA AAC UCU AAG UUU GGA UUU GGG GGC ...3'
Oligo for RNase H activation added
5'... GGU UUC AAC UGC GGA AAC UCU AAG UUU GGA UUU GGG GGC ...3'
3' CCT TTG AGA TTC A AA 5'
5'... GGU UUC AAC UGC GG - - - - UU GGA UUU GGG GGC ...3'
Insertion Duplex Added
5' pA AAC UGU AAG Up 3'
3' AAG TTG ACG CCT TTG ACA TTC AAA CCT AAA CCG 5'
Hybridization of insertion duplex
with deleted mRNA
5'.. GGU UUC AAC UGC GG pA AAC UGU AAG Up UU GGA UUU GGG GGC..3'
3' AAG TTG ACG CC T TTG ACA TTC A AA CCT AAA CCG 5'
Some endonuclelytic catalysis, and repair
Repaired (active) mRNA
5'... GGU UUC AAC UGC GGA AAC UGU AAG UUU GGA UUU GGG GGC ...3'

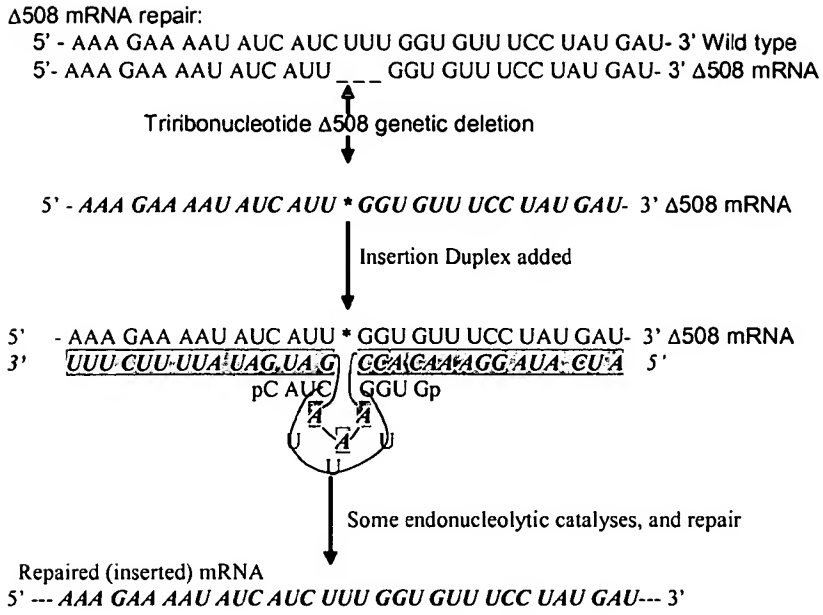
Scheme 11


Inactive mRNA
5'... GGU UUC AAC UGC GGA AAC UcU AAG UUU GGA UUU GGG GGC ...3'
Insertion Duplex Added
C
5'... GGU UUC AAC UGC GGA AAC U U AAG UUU GGA UUU GGG GGC ...3'
3' AAG TTG ACG CCT TTG A A TTC AAA CCT AAA CCC 5'
C
5' pA AAC U UAAG Up 3'
G
Some endonucleolytic catalysis, and repair
Repaired (active) mRNA
5'... GGU UUC AAC UGC GGA AAC UGU AAG UUU GGA UUU GGG GGC ...3'

Scheme 12


Δ508 mRNA repair:
5' - AAA GAA AAU AUC AUC UUU GGU GUU UCC UAU GAU- 3' Wild type
5'- AAA GAA AAU AUC AUU _ _ _ GGU GUU UCC UAU GAU- 3' Δ508 mRNA
Triribonucleotide Δ508 genetic deletion
5' - AAA GAA AAU AUC AUU*GGU GUU UCC UAU GAU- 3' Δ508 mRNA
3' ATAG TAA CCA CAAA 5'
Oligo for RNase H activation added
5' --AAA GAA AAU AUC A _ _ _ _ U GUU UCC UAU GAU- 3'
Plus Insertion duplex
5' -- AAA GAA AAU AUC A _ _ _ _ _ _ _ _ U GUU UCC UAU GAU-3'
3' UUU CUU UUA UAG UAG AAA CCA CAA AGG AUA CUA 5'
5' pC AUC UUU GGU Gp 3'
Some endonucleolytic catalyses, and repair
Repaired (inserted) mRNA
5' --- AAA GAA AAU AUC AUC UUU GGU GUU UCC UAU GAU--- 3'

Scheme 13


Δ508 mRNA repair:
5' - AAA GAA AAU AUC AUC UUU GGU GUU UCC UAU GAU- 3' Wild type
5'- AAA GAA AAU AUC AUU ___ GGU GUU UCC UAU GAU- 3' Δ508 mRNA
Triribonucleotide Δ508 genetic deletion
5' - AAA GAA AAU AUC AUU * GGU GUU UCC UAU GAU- 3' Δ508 mRNA
Insertion Duplex added
5' - AAA GAA AAU AUC AUU * GGU GUU UCC UAU GAU- 3' Δ508 mRNA
3' UUU CUU UUA UAG UA G CCA CAA AGG AUA CU A 5'
pC AUC GGU Gp
Some endonucleolytic catalyses, and repair
Repaired (inserted) mRNA
5' --- AAA GAA AAU AUC AUC UUU GGU GUU UCC UAU GAU--- 3'

Scheme 14

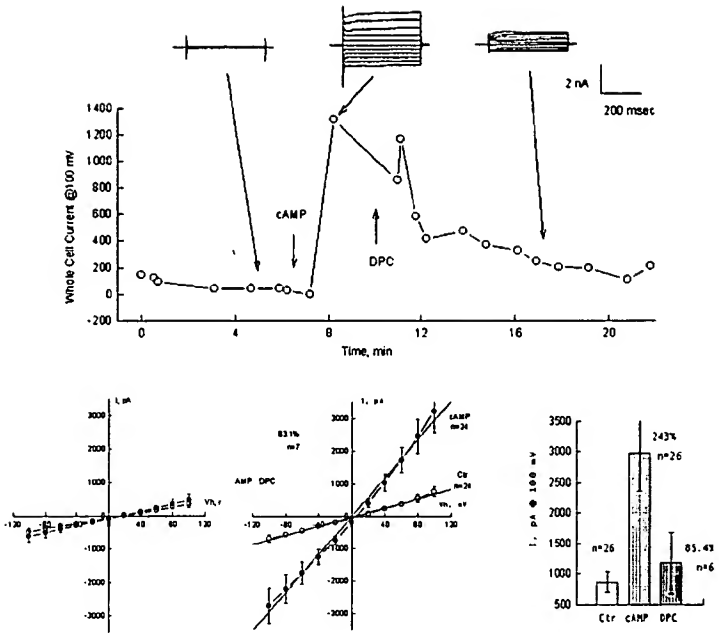


Fig. 1

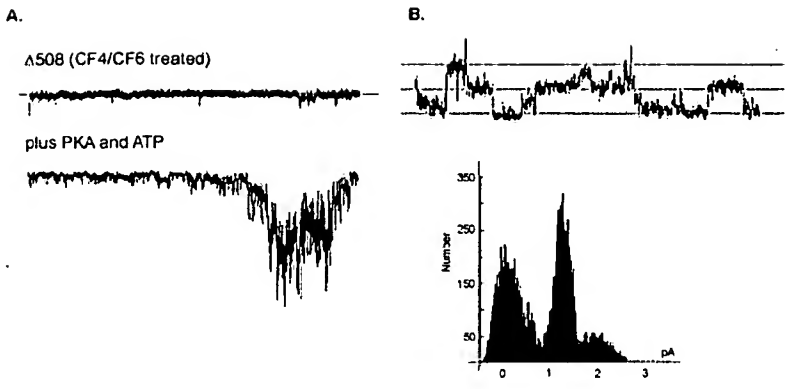

A.
B.
Δ508 (CF4/CF6 treated)
plus PKA and ATP
350
250
150
50
Number
0
1
2
3
pA

Fig. 2

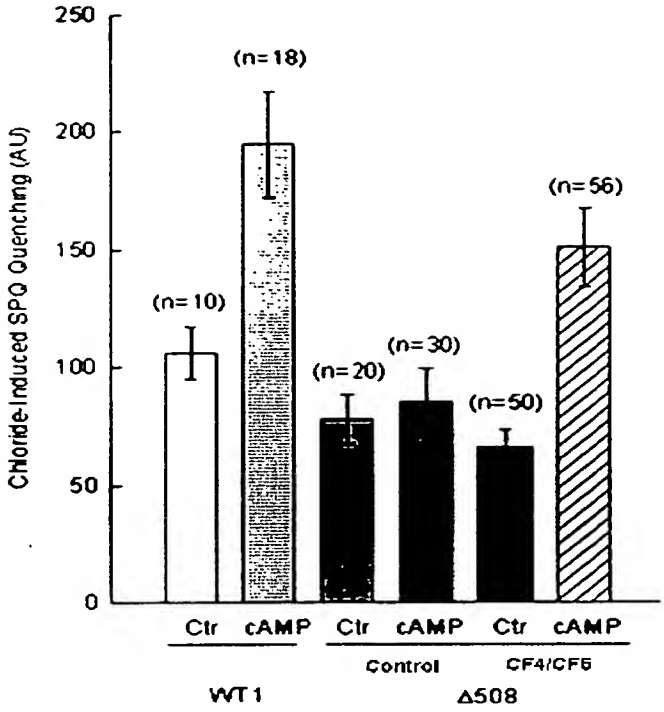

250
200
150
100
50
0
Chloride-Induced SPQ Quenching (AU)
(n=10)
(n=18)
(n=20)
(n=30)
(n=50)
(n=56)
Ctr
cAMP
Ctr
cAMP
Ctr
cAMP
Control
CF4/CF5
WT 1
Δ508

Fig. 3

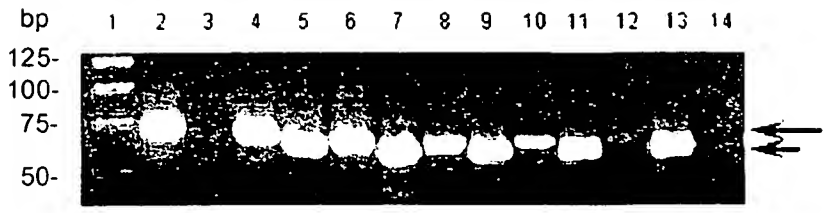


Fig. 4

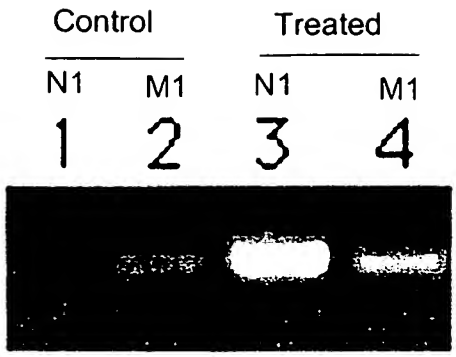


Fig. 5

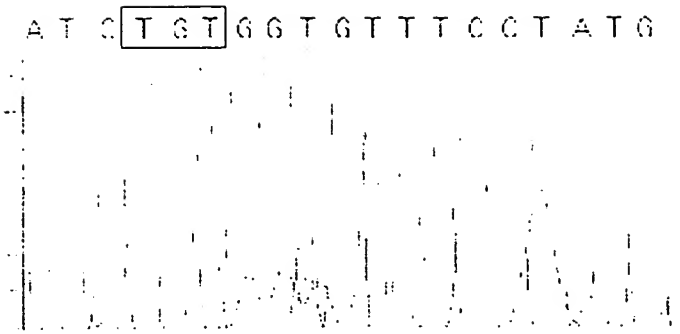


Fig.6